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(57) Abstract

Liver retention clearing agents (LRCAs) and the use thereof are discussed. LRCAs are composed of a hepatic clearance directing component which directs the biodistribution of a LRCA-containing construct to hepatic clearance; a binding component which mediates binding of the LRCA to a compound for which rapid hepatic clearance is desired; a liver retention component which diminishes access of binding component-containing metabolites to target sites; and a structural component to provide a scaffold for the other components.

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LIVER RETENTION CLEARING AGENTS

Technical Field

The present invention relates to liver retention clearing agents (LRCAs), reagents for the preparation thereof and associated methods and compositions. LRCAs impact the elimination and biodistribution of constructs that directly or indirectly become associated with, e.g., incorporate, such agents in a manner resulting in increased elimination via a hepatic route without release of certain metabolites from the liver. The LRCA-associated constructs also generally exhibit a decreased serum half-life in comparison to counterpart compounds which do not incorporate or become associated with LRCAs.

Background of the Invention

Conventional cancer therapy is limited by the problem that the generally attainable targeting ratio (ratio of administered dose of active agent localizing to tumor versus administered dose circulating in blood) is low. This limitation is generally encountered in systemic administration of chemotherapeutic agents as well as in administration of monoclonal antibody-active agent conjugates. Systemic administration involves exposure of healthy tissue to the active agent. Also, as a result of the relatively long half life of a monoclonal antibody, non-target tissue is exposed to circulating antibody-active agent conjugate. Improvement in targeting ratio is therefore sought.

A method employed to improve targeting ratio is referred to generally as pretargeting. In pretargeting, a targeting moiety is formed of a targeting agent and a receptor. The active agent is associated with a ligand for the receptor. The targeting moiety is administered to a recipient, and permitted to localize to the target site with binding at that site mediated by the targeting agent. When target site localization and sufficient elimination of circulating targeting moiety is achieved by the recipient's

metabolism, the active agent-ligand is administered. The ligand component of the construct binds to the pretargeted receptor, thereby delivering the active agent to the target.

Pretargeting is made more efficient by administration of a clearing agent to facilitate elimination of circulating targeting moiety. Various clearing agents have been disclosed. Galactose-human serum albumin (HSA)-biotin clearing agents have been used in pretargeting protocols employing a monoclonal antibody-streptavidin targeting moiety and a biotin-active agent construct. Such clearing agents are discussed in PCT/US93/05406. Derivatization by galactose facilitates elimination of complexes of monoclonal antibody-streptavidin-biotin-HSA-galactose via Ashwell receptors in the liver. These clearing agents rapidly decrease circulating monoclonal antibody-streptavidin levels in patients. Since pretargeting methods are enhanced using clearing agents, improvements in such clearing agents are sought.

Summary of the Invention

The present invention is directed to liver retention clearing agents (LRCAs) which are designed to reduce the level of serum-associated targeting moiety-anti-ligand complex. Preferred LRCAs of the present invention are also designed to prevent release of certain metabolites, i.e., metabolites bearing a ligand or an anti-ligand binding component respectively capable of binding pretargeted anti-ligand or ligand receptor. LRCAs of the present invention are preferably capable of achieving circulating targeting moiety clearance without compromising the binding potential of the pretargeted targeting moiety, either directly by binding of the LRCA thereto or indirectly by binding of LRCA metabolites thereto.

Preferred LRCAs of the present invention also preferably exhibit one or more of the following characteristics:

- rapid, efficient complexation with serum-associated targeting moiety-ligand (or anti-ligand) conjugate *in vivo*;

- rapid clearance from the blood of serum-associated targeting moiety conjugate capable of binding a subsequently administered complementary anti-ligand or ligand containing molecule;

- high capacity for clearing (or inactivating) large amounts of serum-associated targeting moiety conjugate; and

- low immunogenicity.

An additional preferred characteristic of LRCAs of the present invention is operability over a wide LRCA dose range to avoid extensive dose optimization.

Preferred LRCAs of present invention incorporate (1) a hepatic clearance directing component; (2) a binding component; (3) a liver retention component associated with the binding component to promote liver retention of metabolites of LRCA constructs containing ligand or anti-ligand; and (4) a structural component. The structural component serves as a scaffold for binding of the hepatic clearance directing component, the liver retention component and/or the binding component. Preferably the binding component is attached to the structural component through the liver retention component. This construction facilitates the formation of LRCA metabolites containing both the binding component and the liver retention component.

Hepatic clearance directing components of LRCAs of the present invention promote clearance of moieties to which they are attached to the liver. Preferred hepatic clearance directing components include sugar residues recognized by hepatocyte receptors. Preferred LRCAs of the present invention incorporate from about 15 to about 60 sugar residues, with from about 25 to about 50 residues preferred. Also, preferred sugars include galactose and N-acetylgalactosamine. Preferably, the structural component is derivatized with an appropriate number of sugar residues.

Liver retention components of the present invention are designed to prevent release of binding component (such as ligand or anti-ligand)-containing LRCA metabolites to the serum compartment in a manner allowing those metabolites to accrete to pretargeted receptors. Preferably, liver retention components of the present invention are characterized by at least one of the following attributes:

1) Resistance to agents that cleave peptide bonds or otherwise promote catabolism of LRCA-containing moieties;

2) Retention in the cytoplasmic or a subcellular compartment following internalization into hepatic cells; or

3) Excretion upon metabolism without re-entry, or with delayed or retarded re-entry, into the serum compartment (e.g., biliary excretion without reabsorption in the intestine).

Protease-resistant liver retention components of the present invention are useful because formation of binding component-containing metabolites of LRCA constructs capable of accessing target-associated ligand or anti-ligand is not favored. That is, any such metabolites are unlikely to accrete from hepatocytes in a manner permitting access to pretargeted receptors. In a preferred embodiment of the present invention, the ligand or anti-ligand binding component of the LRCA is generally retained in hepatocytes for a time sufficient to allow active agent-ligand or active agent-anti-ligand construct to reach and bind to the pretargeted receptor therefor. An example of a protease-resistant liver retention agent is a poly-amino acid of unnatural (D) orientation. Preferred liver retention components of this type are linear chains of from about 2 to about 12 amino acids and the like. Another example of a metabolism-resistant liver retention component incorporates at least one tertiary amide (-CO-N(R)-) bond, wherein R is preferably lower alkyl. Such bonds are more highly resistant to hydrolytic enzymatic activity (e.g., biotinidase activity) than secondary amide (peptide, -CO-NH-)bonds.

Liver retention moieties that are characterized by a limited capacity to traverse hydrophobic cellular or subcellular membranes also afford enhanced retention of binding component-containing LRCA metabolites in hepatocytes. Consequently, metabolites having the potential to bind to pretargeted receptor are retained in the liver for a time sufficient to permit later administration and accretion to targeted receptor of active agent-containing conjugate. Diffusion-restricted liver retention moieties of the present invention are sufficiently polar or sufficiently charged to render passage through the non-polar lipid bilayer membrane structures difficult. Examples of such liver retention components are moieties characterized by positive charge, negative charge or neutral charge combined with hydrophilicity. Preferred liver retention components of this type are saccharides (neutral charge/hydrophilic), phosphates and phosphonates (negative charge), polylysines (positive charge), polyglutamic acids (negative charge) and the like.

Liver retention component-binding component metabolites that are characterized by relatively rapid excretion, without passage into the serum compartment, are also useful in the practice of the present invention. Metabolites excreted by a hepatobiliary route without reabsorption by the intestines are preferred for use in this aspect of the present invention. Examples of rapid excretion-liver retention components are pepstatin, 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetra acetic acid (DOTA) and the like. Pepstatin is rapidly excreted into the bile. When internalized into hepatocytes, DOTA is generally either trapped therein or excreted via a hepatobiliary route.

Another group of moieties useful as liver retention components in the practice of the present invention are moieties employed in the prior art to retain radioactivity at tumor target sites. These molecules also afford resistance to enzymatic degradation. Examples of such liver retention components are cellobiose, dilactitol, lysyl-epsilon-amido 5-iodo-3-pyridinecarboxylate, other non-mammalian sugars, other radiolabel residualizing moieties and the like.

Liver retention components useful in the present invention may combine more than one of the desirable properties set forth above. Preferred LRCAs of the present invention are characterized by the following liver retention components:

- poly-(D) orientation lysine residues (metabolic stability and positive charge);
- poly- (D) orientation glutamic acid residues (metabolic stability and negative charge);
- poly- galactose-derivatized (D) orientation lysine residues (metabolic stability and polarity); and
- poly-phosphonate, for example, based upon alpha-phosphonomethyl amino acid compounds, such as the following:

$HOOC\text{-}CH(NH_2)CH_2\text{-}P(OH)_2$ (negative charge).

Each of these preferred liver retention components incorporate from about 2 to about 12 monomers, with from about 3 to about 6 monomers, more preferred. Also preferred as liver retention components in the practice of the present invention are polymers of natural amino acids of the (L) configuration employed in combination

with a tertiary amide of a biotin binding moiety.

Binding components of the LRCAs of the present invention are moieties which recognize epitopes or components of molecules to be cleared by the LRCAs. Preferred binding components of LRCAs of the present invention are members of ligand/anti-ligand pairs or lower affinity forms thereof that facilitate binding to targeting moiety-anti-ligand/ligand conjugate. A preferred ligand/anti-ligand pair for use in the practice of the present invention is biotin-avidin. Preferred LRCAs of the present invention incorporate from about 1 to about 10 ligand or anti-ligand molecules, with from about 1 to about 4 more preferred and with from about 1 to about 2 still more preferred.

Preferred structural components include proteinaceous and non-proteinaceous materials having sufficient reactive groups for derivatization with hepatic clearance directing components and binding components and/or liver retention components, such as proteins and polymers. More preferred proteinaceous structural components include those expected to elicit little response from a recipient's immune system. Consequently, human proteins, such as human serum albumin, IgG, IgM and the like constitute structural components useful in the practice of the present invention. Preferred polymeric structural components of the present invention include dextran, hydroxypropylmethylacrylamide (HPMA), hydroxypropylacrylamide, hydroxypropylethylacrylamide, poly-D-lysine, poly-D-glutamate, poly-D-aspartate, dendrimers (spherical constructs having functional units on the exterior thereof) and the like.

Brief Description of the Drawings

Figure 1 illustrates the tumor uptake profile of antibody-streptavidin conjugate (Ab/SA) in comparison to a control profile of native whole antibody (Ab) and streptavidin (SA).

Figures 2a and 2b schematically illustrate the preparation of a liver retention component-binding component construct, N-methyl-N-{5'-[methylester tris-(-(D, L)-phosphonoalanyl)-(D)-cystyl]-5-carbamylpentyl}biotinamide.

Figures 3a and 3b schematically illustrate the preparation of a liver retention

component-binding component construct, N-methyl-N-[5-(triglutamylcysteine)-5-carbamylpentyl]-biotinamide.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to set forth definitions of certain terms to be used within the disclosure.

Targeting moiety: A molecule that binds to a defined population of cells. The targeting moiety may bind a receptor, an oligonucleotide, an enzymatic substrate, an antigenic determinant, or other binding site present on or in the target cell population. Antibody is used throughout the specification as a prototypical example of a targeting moiety. Tumor is used as a prototypical example of a target in describing the present invention.

Ligand/anti-ligand pair: A complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity. Exemplary ligand/anti-ligand pairs include zinc finger protein/dsDNA fragment, enzyme/inhibitor, hapten/antibody, lectin/carbohydrate, ligand/receptor, S-protein/S-peptide, head activator protein (which binds to itself), cystatin-C/cathepsin B, and biotin/avidin. Biotin/avidin is used throughout the specification as a prototypical example of a ligand/anti-ligand pair.

Anti-ligand: As defined herein, an "anti-ligand" demonstrates high affinity, and preferably, multivalent binding of the complementary ligand. Preferably, the anti-ligand is large enough to avoid rapid renal clearance, and is multivalent to bind a larger number of ligands. Univalent anti-ligands are also contemplated by the present invention. Anti-ligands of the present invention may exhibit or be derivatized to exhibit structural features that direct the uptake thereof, e.g., galactose residues that direct liver uptake. Avidin and streptavidin are used herein as prototypical anti-ligands.

Avidin: As defined herein, "avidin" includes avidin, streptavidin and derivatives and analogs thereof that are capable of high affinity, multivalent or univalent binding of biotin.

Ligand: As defined herein, a "ligand" is a relatively small, soluble molecule that

binds with high affinity by anti-ligand and preferably exhibits rapid serum, blood and/or whole body clearance when administered intravenously in an animal or human. Biotin constructs are used as prototypical ligands.

Lower Affinity Ligand or Lower Affinity Anti-Ligand: A ligand or anti-ligand that binds to its complementary ligand-anti-ligand pair member with an affinity that is less than the affinity with which native ligand or anti-ligand binds the complementary member. Preferably, lower affinity ligands and anti-ligands exhibit between from about 10^{-6} to 10^{-10} M binding affinity for the native form of the complementary anti-ligand or ligand. For avidin/streptavidin and other extremely high affinity binding molecules , however, lower affinity may range between 10^{-6} to 10^{-13} M. Lower affinity ligands and anti-ligands may be employed in clearing agents of the present invention.

Active Agent: A diagnostic or therapeutic agent ("the payload"), including radionuclides, drugs, anti-tumor agents, toxins, superantigens and the like. Radionuclide therapeutic agents are used as prototypical active agent. Attachment of such radionuclide active agents to other moieties, either directly or via chelation technology, may be accomplished as described herein or as known in the art.

Pretargeting: As defined herein, pretargeting involves target site localization of a targeting moiety that is conjugated with one member of a ligand/anti-ligand pair; after a time period sufficient for optimal target-to-non-target accumulation of this targeting moiety conjugate, active agent conjugated to the opposite member of the ligand/anti-ligand pair is administered and is bound (directly or indirectly) to the targeting moiety conjugate at the target site (two-step pretargeting). Three-step and other related methods described herein are also encompassed.

Clearing Agent: An agent capable of binding, complexing or otherwise associating with an administered moiety (e.g., targeting moiety-ligand, targeting moiety-anti-ligand or anti-ligand alone) present in the recipient's circulation, thereby facilitating circulating moiety clearance from the recipient's body, removal from blood circulation, or inactivation thereof in circulation. The clearing agent is preferably characterized by physical properties, such as size, charge, reduced affinity, configuration or a combination thereof, that limit clearing agent access to the

population of target cells recognized by a targeting moiety used in the same treatment protocol as the clearing agent.

Conjugate: A conjugate encompasses chemical conjugates (covalently or non-covalently bound), fusion proteins and the like.

Liver Retention Clearing Agent (LRCA): A moiety capable of directing the clearance of a moiety to which it is bound upon administration or of a component to which it becomes associated with in vivo. LRCAs of the present invention direct clearance via a hepatic pathway. Preferred LRCAs of the present invention are characterized by a structural component, a hepatic clearance directing component, a liver retention component and a binding component.

Hepatic Clearance Directing Component: A plurality of sugar residues recognized by a liver receptor. Hepatic clearance directing components preferably contain from 15 about 60 sugar residues, with from 25 to about 50 sugar residues preferred. The structural component is preferably derivatized with an appropriate number of sugar residues.

Liver Retention Component: A moiety designed to prevent release of ligand- or anti-ligand-containing metabolites of LRCA constructs to the serum compartment in a manner allowing those metabolites to accrete to pretargeted receptors. Preferable liver retention components of the present invention are characterized by at least one of the following attributes:

1) Resistance to agents that cleave peptide bonds or otherwise promote catabolism of LRCA-containing moieties;

2) Retention in the cytoplasmic or a subcellular compartment following internalization into hepatic cells; or

3) Excretion upon metabolism without re-entry, or with delayed or retarded re-entry, into the serum compartment.

Binding Component: A ligand, anti-ligand or other moiety capable of in vivo association with a previously administered molecule (bearing the complementary ligand or anti-ligand, for example) or with another toxic or potentially toxic molecule present in the recipient's circulation or extravascular fluid space via recognition by the binding component of an epitope associated with the previously administered moiety

or with the toxic or potentially toxic molecule.

Structural Component: A moiety which serves as a scaffold for binding of the hepatic clearance directing component, the liver retention component and, optionally, the binding component. Preferred structural components include proteinaceous and non-proteinaceous materials having sufficient reactive groups for such derivatization, such as human proteins and polymers.

The LRCAs of the present invention are preferably employed in pretargeting protocols. "Two-step" pretargeting procedures feature targeting moiety-ligand or targeting moiety-anti-ligand (targeting moiety-receptor) administration, followed by administration of active agent conjugated to the opposite member of the ligand-anti-ligand pair. As step "1.5" in the two-step pretargeting methods of the present invention, a LRCA is administered to facilitate the clearance of circulating targeting moiety-receptor conjugate.

In the two-step pretargeting approach, the clearing agent preferably does not become bound to the target cell population, either directly or through the previously administered and target cell bound targeting moiety-anti-ligand or targeting moiety-ligand conjugate. An example of two-step pretargeting involves the use of biotinylated human transferrin as a clearing agent for avidin-targeting moiety conjugate, wherein the size of the clearing agent results in liver clearance of transferrin-biotin-circulating avidin-targeting moiety complexes and substantially precludes association with the avidin-targeting moiety conjugates bound at target cell sites. (See, Goodwin, D.A., Antibod. Immunoconj. Radiopharm., 4: 427-34, 1991).

LRCAs of the present invention contain a hepatic clearance directing component, a liver retention component, a binding component and a structural component. Thus, LRCAs of the present invention are bispecific in that the hepatic clearance directing component mediates interaction with heptocyte receptors and the binding component mediates binding with the moiety to be cleared. These bispecific LRCAs are capable of *in vivo* binding or association with molecules to be cleared and interaction with hepatic receptors to effect clearance of LRCA-containing constructs by that route. Preferred LRCAs of the present invention are suitable for use as a

clearing agent in pretargeting protocols, including two step protocols.

Clearing agents useful in the practice of the present invention preferably exhibit one or more of the following characteristics:

- rapid, efficient complexation with serum-associated targeting moiety-ligand (or anti-ligand) conjugate in vivo;

- rapid clearance from the blood of serum-associated targeting moiety conjugate capable of binding a subsequently administered complementary anti-ligand or ligand containing molecule;

- high capacity for clearing (or inactivating) large amounts of serum-associated targeting moiety conjugate; and

- low immunogenicity.

Clearing agents previously developed by the assignee of this patent application incorporated, for example, a structural component of human serum albumin (HSA), a plurality of hexoses and a plurality of ligands, as follows:

$(Hexose)_m$--Human Serum Albumin (HSA)--$(Ligand)_n$,

wherein n is an integer from 1 to about 10 and m is an integer from 1 to about 45 and wherein the hexose is recognized by liver receptors, e.g., Ashwell receptors.

The exposed hexose residues direct the clearing agent to rapid clearance by endocytosis into the liver through specific receptors therefor. These receptors bind the clearing agent or clearing agent-containing complexes, and induce endocytosis into the hepatocyte, leading to fusion with a lysosome and recycling of the receptor back to the cell surface. This clearance mechanism is characterized by high efficiency, high capacity and rapid kinetics. The rapid kinetics of hexose-mediated liver uptake, coupled with a relatively high affinity interaction between the binding moiety, such as a ligand, and the compound to be cleared, provide for rapid and efficient clearance.

LRCAs of the present invention are designed to meet the four criteria set forth above as well. Two additional performance criteria were instituted for LRCAs:

- operability over a wide LRCA dose range to avoid the desirability of extensive dose optimization; and

- low ability to compromise pretargeted receptor.

Preferred LRCAs of the present invention are therefore characterized by a liver

retention component which diminishes the ability of LRCA metabolites, particularly binding component-containing metabolites, to accrete to pretargeted receptor. This feature also serves to increase the dose range over which the LRCA may be employed, because compromise of pretargeted receptor by such binding component-containing metabolites is decreased or avoided. Preferred LRCAs of the present invention also incorporate a structural component of proteinaceous or non-proteinaceous composition. Such preferred LRCAs exhibit physical properties facilitating use for in vivo complexation and blood clearance of anti-ligand/ligand-targeting moiety conjugates.

Other embodiments of the present invention involve the preparation and use of LRCAs in clearance of other previously administered molecules or toxic or potentially toxic molecules generated *in vivo*, which compounds to be cleared are present in a patient's circulation or extravascular fluid space. Previously administered molecules may include active agent-containing conjugates (e.g., radionuclide-chelate-antibody which can be cleared by a LRCA containing an anti-chelate or anti-antibody binding moiety; or radionuclide-chelate-antibody-biotin binding protein which can be cleared by a biotin-containing LRCA); targeting moiety-receptor conjugates; or the like.

Preferred LRCAs of the present invention are administered and permeate the circulation. Consequently, previously administered compounds or toxic or potentially toxic moieties that are present in the circulation are accessible to the LRCAs of the present invention. Circulating compounds are removed from the serum via association with the LRCA and processing by liver receptors. Previously administered compounds or toxic or potentially toxic moieties, present in extravascular fluid space but not associated with a target cell or epitope, are removed by the LRCAs of the present invention via liver receptors as such compounds diffuse back into the circulation and become associated with LRCAs. Toxic or potentially toxic molecules that may be removed from a recipient's circulation or extravascular fluid space include: chemotherapeutics e.g., alkylators, heavy metals and the like.

Binding components useful in the practice of the present invention are capable of associating with the molecule to be cleared. Suitable binding components therefore include those moieties that are capable of associating with toxic or potentially toxic

molecules present in the recipient's circulation, which include antibodies or fragments thereof directed to epitopes that are characteristic of such toxin or potential toxin. Other useful binding components include oligonucleotides, ligands or anti-ligands. Ligands and anti-ligands are preferred binding components of the present invention. A particularly preferred binding component for use in the practice of the present invention is biotin or a derivative or analog thereof.

Characteristics of useful binding components are discussed below. The binding between the binding component of the LRCAs of the present invention and the molecule to be cleared from the circulation need only be transient, i.e., exists for a sufficient amount of time to clear the molecule to the liver and for hepatocyte internalization. Also, it should be noted that the binding constant of the binding component is determined with regard to the LRCA as a whole. That is, a biotin-containing LRCA is expected to bind to avidin or streptavidin with a binding constant less than that of biotin itself. Experimentation has revealed that the LRCAs of the present invention are capable of clearance.

In general, the binding constant must be sufficiently high to capture the molecule to be bound and traffic that molecule to the liver for internalization into hepatocytes. Consequently, LRCA binding components having a binding constant in excess of about 10^8 are preferred.

For use in an LRCA of the present invention, the number of binding components ranges from about 1 to about 10, and preferably from about 1 to about 4 and more preferably from about 1 to about 2.

Binding components of the present invention include ligands, anti-ligands, and other target epitope-recognizing moieties. One skilled in the art can substitute acceptable moieties for the binding components discussed specifically herein. Preferred binding components are characterized by a molecular weight of a Fab fragment of a monoclonal antibody or lower. Such binding components may also be modified to include suitable functional groups to allow for attachment of other molecules of interest, e.g., peptides, proteins, nucleotides, and other small molecules.

LRCAs of the present invention are designed to interact with hepatic receptors to facilitate clearance of LRCA-containing constructs via that route. The hepatic

clearance directing component of the LRCA is included for this purpose.

Hepatocyte receptors which provide for effective clearance include in particular Ashwell receptors, mannose receptors associated with endothelial cells and/or Kupffer cells of the liver, the mannose 6-phosphate receptor, and the like. Hexoses which may be employed in the LRCA structure include by way of exampie galactose, mannose, mannose 6-phosphate, N-acetylgalactosamine, pentamannosyl-phosphate, and the like. Hexoses recognized by Ashwell receptors include glucose, galactose, galactosamine, N-acetylgalactosamine, pentamannosyl phosphate, mannose 6-phosphate and thioglycosides of galactose, galactosides, galactosamine, and N-acetylgalactosamine. A sufficient number of hexose residues are incorporated into the LRCA to provide for effective clearance, e.g., via the Ashwell receptors located on the surface of hepatocytes.

Preferable hepatic clearance directing components of this embodiment of the LRCAs of the present invention constitute between about 15 and about 60 hexose residues, e.g., galactose residues or N-acetylgalactosamine residues. More preferably hepatic clearance directing components of the present invention constitute between about 25 and 50 hexose residues. Preferably, the LRCA structural component is derivatized by an appropriate number of sugar residues. However, the invention is not limited thereby and embraces the attachment of any number of hexose residues or mixture thereof which results in an efficacious bispecific LRCA.

Liver retention components of the present invention are designed to prevent release of binding component-containing metabolites of LRCA constructs to the serum compartment in a manner allowing those metabolites to accrete to pretargeted receptors. Preferably, liver retention components of the present invention are characterized by at least one of the following attributes:

1) Resistance to agents that cleave peptide bonds or otherwise promote the catabolism of LRCA-containing moieties;

2) Retention in the cytoplasmic or a subcellular compartment following internalization into hepatic cells; or

3) Excretion upon metabolism without re-entry, or with delayed or retarded re-entry, into the serum compartment.

Protease-resistant liver retention components of the present invention are useful, because formation of binding component-containing metabolites of LRCA constructs capable of accessing target-associated ligand or anti-ligand is not favored. That is, any such metabolites are unlikely to accrete from hepatocytes in a manner permitting access to pretargeted receptors. In a preferred embodiment of the present invention, the ligand or anti-ligand binding component of the LRCA is generally retained in hepatocytes for a time sufficient to allow active agent-ligand or active agent-anti-ligand construct accretion to target.

An example of a protease-resistant liver retention agent is a poly-amino acid of unnatural (D) orientation. The (D) amino acid sequence provides resistance to catabolic processing, because lysosomal exopeptidases and endopeptidases recognize peptides of the natural (L) orientation. Thus, the (D) amino acids constitute a poor substrate for the peptidases. Preferred liver retention components of this type are linear chains of from about 2 to about 12 (D) amino acids and the like, with from about 3 to about 10 (D) amino acids preferred. It should be noted, however, that more than 12 (D) amino acids could be employed as well. Preferred poly (D) amino acids are charged (D) amino acid polymers, such as poly (D) lysine, poly (D) glutamic acid, poly (D) aspartate, poly (D) ornithine and the like. Other protease-resistant liver retention agents useful in the practice of the present invention include the following: alpha-aminoisobutyric acid (AIB) and N-alkyl-substituted amino acids which form tertiary amide bonds rather than secondary amide (peptide) bonds. The alkyl moiety is lower alkyl, preferably methyl, ethyl, propyl or butyl, with methyl most preferred. Alternatively, the tertiary amide can be formed using an N-phosphono substitution.

Liver retention moieties that are characterized by a limited capacity to traverse hydrophobic cellular or subcellular membranes also afford enhanced retention of binding component-containing metabolites of LRCA-containing constructs in hepatocytes. Consequently, metabolites having the potential to bind to pretargeted receptor are retained in the liver for a time sufficient to permit accretion to targeted receptor of active agent-containing conjugate.

Diffusion-restricted liver retention moieties of the present invention are sufficiently polar or sufficiently charged to render passage through non-polar lipid

bilayer membranes difficult under hepatocyte cellular conditions, pH 6-7. Examples of such liver retention components are moieties characterized by positive charge, negative charge or neutral charge combined with hydrophilicity. Preferred liver retention components of this type are saccharides (neutral charge/hydrophilic), phosphates or phosphonates (negative charge), polylysines (positive charge), polyglutamic acids (negative charge) and the like.

With regard to subcellular membranes, diffusion-restricted liver retention components are sufficiently polar or sufficiently charged to render passage through the non-polar lipid bilayer membranes difficult under subcellular (e.g., lysosomal) conditions. For example, lysosomes are characterized by a pH of about 5. Thus, moieties expected to be highly charged at acidic pH (basic moieties) are desirable for use as liver lysosomal retention components. Also, basic proteins are generally catabolized more slowly than acidic proteins. As a result, liver retention components made up of basic amino acids, such as lysine, histidine, arginine, ornithine and the like, are useful lysosomal membrane, diffusion-resistant liver retention components of the present invention.

Positively charged, diffusion-resistant liver retention agents useful in the practice of the present invention include the following: polylysines, polyhistidines, polyarginines and the like.

Negatively charged, diffusion-resistant liver retention agents useful in the practice of the present invention include the following: polyglutamic acids, polyphosphates, polyphosphonates, such as poly-alpha-phosphonomethyl amino acids, polyaspartates and the like.

Neutral charge/hydrophilic, diffusion-resistant liver retention agents useful in the practice of the present invention include the following: disaccharides such as cellobiose and lactose, deoxysorbitol, dilactitol, amino-naphthaltyrimide-deoxysorbitol (ANTDS), unnatural polysaccharides, D-poly amino acid saccharide derivatives and the like. See, for example, Ali et al., "Synthesis and Radioiodination of Tyramine Cellobiose for Labeling Monoclonal Antibodies," Nucl. Med. Biol., 15(5): 557-61, 1988, and Demignot et al., "Differences between the catabolism and tumour distribution of intact monoclonal antibody (791T/36) and its Fab/c fragment in mice

with tumour xenografts revealed by the use of a residualizing radiolabel (dilactitol-^{125}-I-tyramine) and autoradiography," Cancer Immunol. Immunother., 33: 359-66, 1991.

For liver retention components involving poly-amino acids, the number of such amino acids is selected to prevent passive diffusion of the binding component-liver retention component metabolite of the LRCA from hepatocyte lysosomes. Literature indicates that certain dipeptides diffuse freely, while tripeptides generally do not. Consequently, liver retention components having three or more amino acids are generally preferred. Also, lengthy poly-amino acids present some synthetic challenges. Thus, liver retention components having 12 or fewer amino acids are generally preferred.

Liver retention component-binding component metabolites that are characterized by relatively rapid excretion, without passage into the serum compartment are also useful in the practice of the present invention. Metabolites excreted by a hepatobiliary route without reabsorption by the intestines are preferred for use in this embodiment of the present invention. Examples of excretion-liver retention components are pepstatin, 1,4,7,10-tetraazacyclododecane-N,N',N",N'"-tetra acetic acid (DOTA) and the like. Pepstatin is rapidly excreted into the bile. DOTA is excreted via a hepatobiliary route following internalization by hepatocytes. Other rapid excretion liver retention agents useful in the practice of the present invention include the following: diethylene triamine penta-acetic acid (DTPA), ethylene diamine tetra-acetic acid (EDTA), ethylene glycol bis-(beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and the like.

Another group of moieties useful as liver retention components in the practice of the present invention are moieties employed in the prior art to retain radioactivity at tumor target sites. The present inventors believe that retention following internalization by hepatocytes will operate similarly to retention upon internalization by tumor cells. In tumor and hepatocyte cells, retention can be generated by a combination of lysosomal/intracellular retention and decreased susceptibility to metabolic degradation. Examples of such liver retention components are cellobiose, dilactitol, lysyl-epsilon-amido 5-iodo-3-pyridinecarboxylate, other non-mammalian

sugars, other radiolabel residualizing moieties and the like.

Liver retention components useful in the present invention may combine more than one of the desirable properties set forth above. Preferred LRCAs of the present invention are characterized by the following liver retention components:

- poly-(D) orientation lysine residues (metabolic stability and positive charge);

- poly- (D) orientation glutamic acid residues (metabolic stability and negative charge);

- poly- galactose-derivatized (D) orientation lysine residues (metabolic stability and polarity); and

- poly-phosphonate, for example, based upon alpha phosphonomethyl amino acids, such as

$HOOC\text{-}CH(NH_2)CH_2\text{-}P(OH)_2$ (negative charge).

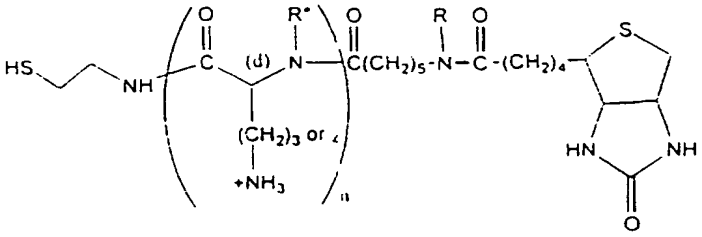
Amino acids of natural (L) configuration may be employed in the preferred liver retention components; provided that at least one bond in the liver retention component-binding moiety construct is a tertiary amide bond. Such tertiary amide bonds are resistant to enzymatic degradation. The stabilized bond should be incorporated in the construct, such that the biding component remains associated with a sufficient portion of the liver retention component to prevent access of the binding moiety to pretargeted receptor.

When a biotin binding moiety, for example, is employed in a LRCA of the present invention, polyamino acids of natural (L) orientation may be employed, provided that the amide bonds between the biotin and the polyamino acid and between the individual amino acids of the polyamino acid incorporate a tertiary amine. The polyamino acids serve to prevent egress of biotin-containing metabolites to pretargeted avidin or streptavidin. The biotinidase-resistant tertiary amide ensures that biotin will remain associated with the polyamino acid.

Each of these preferred liver retention components incorporate from about 2 to about 12 monomers, with from about 3 to about 6 monomers, more preferred.

Preferably the binding component is attached to the structural component through the liver retention component. This construction facilitates the formation of

LRCA metabolites containing both the binding component and the liver retention component. Examples of specific preferred embodiments of biotin binding component/liver retention component combinations for use in LRCAs of the present invention are shown below:



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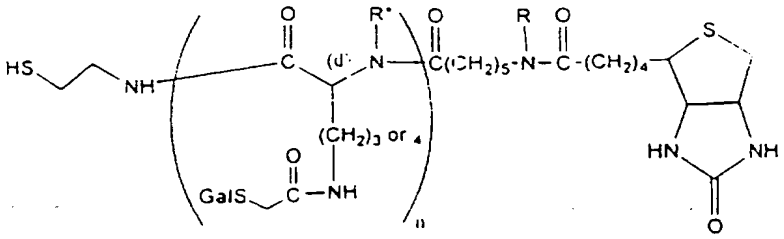

(2)
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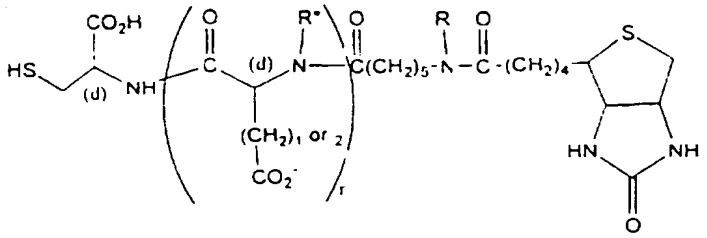
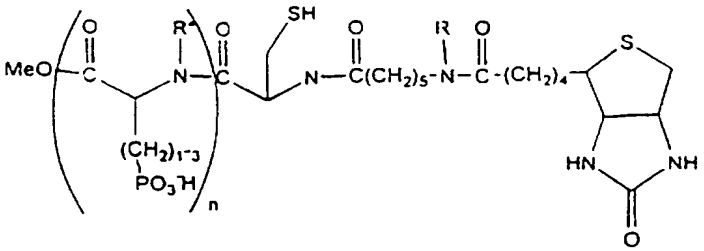


(3)
$(d-glu)_n$ BT



(4)
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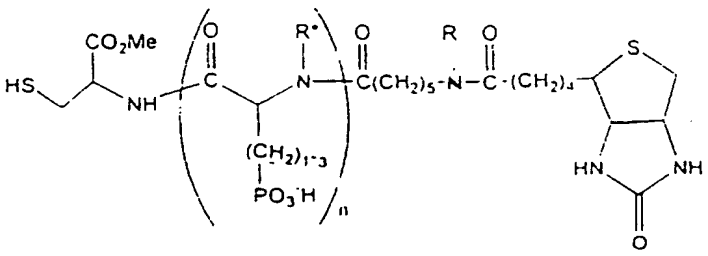


(5)
(phos)$_n$ BTII

where n is 1 to 50, preferably about 3 to about 12, and more preferably from about 3 to about 6; R is a lower alkyl moiety of from 1 to about 6 carbon atoms; and R* is hydrogen or lower alkyl from 1 to about 6 carbon atoms.

Biotinidase generally cleaves a secondary amide bond adjacent to the biotin. In conducting research relating to active agent constructs involving DOTA-biotin, it was discovered that substitution of the amide nitrogen with, for example, an alkyl moiety resulted in stability with respect to biotinidase cleavage. Consequently, preferred liver retention component-biotin constructs employ a tertiary amide nitrogen bearing an alkyl substituent, preferably of from 1 to about 6 carbon atoms and more preferably of from 1 to about 4 carbon atoms.

LRCAs of the present invention also contain a structural component of proteinaceous or non-proteinaceous composition. Preferred structural components are characterized by or are derivatized to contain sufficient reactive groups for binding with hepatic clearance directing components and binding components and/or liver retention components. Consequently, such structural components must incorporate from about 16 to about 70 functional groups, and more preferably from about 26 to about 52 functional groups. These ranges are derived as follows: Preferred hepatic clearance directing component derivatization is from about 15 to about 60 hexoses (more preferred, from about 25 to about 50); and preferred binding component/liver retention component derivatization is from about 1 to about 10

(most preferred, from about 1 to about 2).

Derivatization with both hexose and binding component are conducted in a manner sufficient to produce individual clearing agent molecules with a range of derivatization levels that averages a recited whole number. For example, biotinylation levels of a LRCA average a recited whole number, such as 1, biotin. Derivatization of a structural component with 3 equivalents of biotin, for example, produces a product mixture made up of individual LRCAs, substantially all of which having at least one biotin residue. Derivatization with 1 biotin equivalent produces a LRCA product mixture, wherein a significant portion of the individual molecules are not biotin derivatized. The whole numbers used in this description refer to the average derivatization of the LRCAs under discussion.

One embodiment of LRCA of the present invention incorporates a proteinaceous structural component of intermediate molecular weight (ranging from about 40,000 to about 200,000 Dal), such as asialoorosomucoid, human serum albumin or other soluble natural protein, preferably those having low immunogenicity when administered to humans. Alternatively, LRCAs may include polyglutamate, polylysine, polyarginine, polyaspartate and like structural components. High molecular weight structural components(ranging from about 200,000 to about 1,000,000 Dal) characterized by poor target access, including IgM or IgG (approximately 150,000 Dal) molecules, ferritin (approximately 445 kD) may also be employed. Chemically modified polymers of intermediate or high molecular weight (ranging from about 40,000 to about 1,000,000 Dal), such as dextran, hydroxypropylmethacrylamide polymers, polyvinylpyrrolidone-polystyrene copolymers, divinyl ether-maleic acid copolymers, pyran copolymers, or polyethylene glycol (PEG), also have utility as structural components of LRCAs of the present invention. In addition, liposomes (high molecular weight moieties with poor target access) can be used as a structural component of LRCAs of the present invention.

LRCAs having a human protein as the structural component thereof are preferred for use in the practice of the present invention. Human proteins, especially human serum proteins, such as, for example, orosomucoid and human serum albumin, human IgG, human-anti-antibodies of IgG, IgA and IgM class, and the like, are less

immunogenic upon administration into the serum of a human recipient. Human orosomucoid is commercially available from, for example, Sigma Chemical Co. St. Louis, Missouri. Treatment of orosomucoid with neuraminidase removes sialic acid residues, thereby exposing galactose residues, forming asialoorosomucoid. Human HSA (Cutter Biological) and human IgG, IgA and IgM (Sigma Chemical Co.), for example, are also commercially available. Other proteinaceous structural components include albumin, IgM, IgG, asialohaptoglobin, asialofetuin, asialoceruloplasmin and the like.

Human serum albumin is a preferred proteinaceous structural component for LRCAs of the present invention. Other mammalian forms of human serum albumin, which differ from human serum albumin only by a few amino acid residues, may also be used in the practice of the present invention. Examples of such mammalian forms of serum albumin are bovine serum albumin, porcine serum albumin, and the like.

In general, LRCAs of the present invention are prepared in the following manner:

1) The hepatic clearance directing component is conjugated to the structural component;

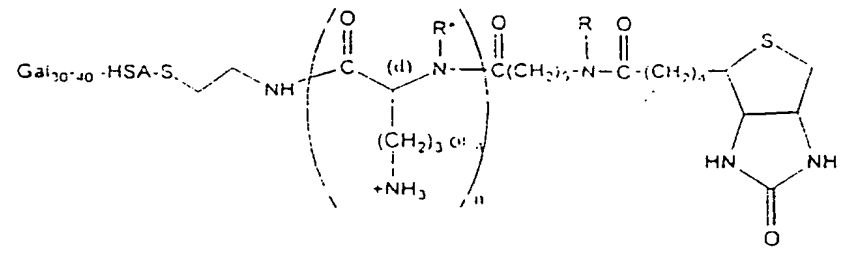
2) The hepatic clearance directing component-structural component construct is derivatized, preferably with from about 1 to about 10, more preferably from about 1 to about 4, and still more preferably from about 1 to about 2 reactive groups;

3) Binding component/liver retention component constructs are synthesized and are either characterized or are derivatized to contain a complementary reactive group to those generated in step (2); and

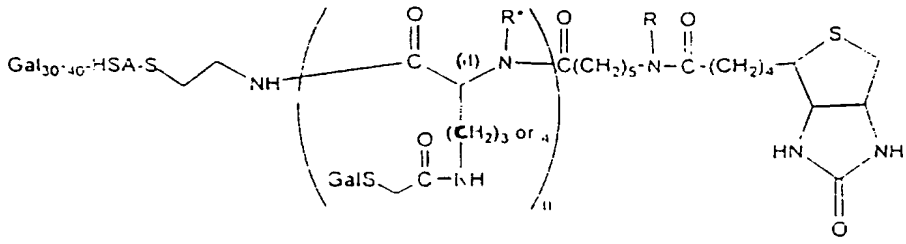
4) Binding component/liver retention component is conjugated to hepatic clearance directing component-structural component to form an LRCA.

The reactive groups set forth herein are such groups as are generally employed in organic synthesis. Complementary reactive groups are well known to those skilled in the art and include, for example, maleimide-sulfhydryl, active ester-amine, isothiocyanate-amine and the like. Selection of useful reactive groups is within the ordinary skill in the art.

Preferred LRCAs of the present invention are shown below:



(1)
HSA (d-lys)$_n$ BT



(2)
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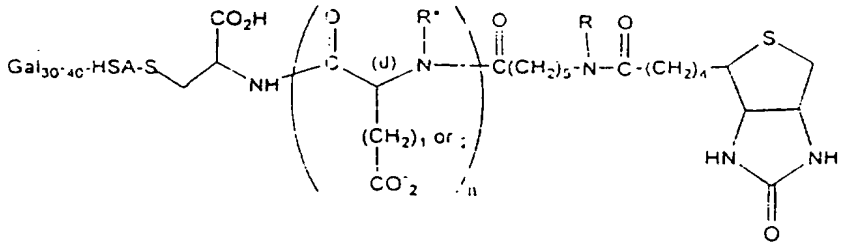

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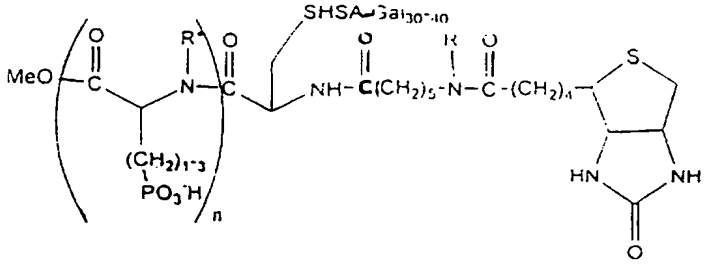

(4)
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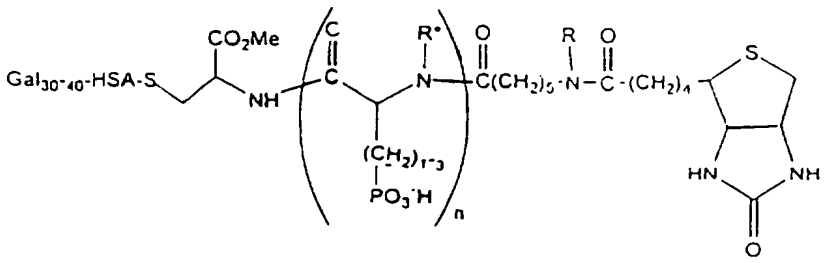


(5)
HSA (phos)$_n$ BTII

where n is 1 to 50, preferably about 3 to about 12, and more preferably from about 3 to about 6; R is a lower alkyl moiety of from 1 to about 6 carbon atoms; and R^* is hydrogen or lower alkyl from 1 to about 6 carbon atoms.

Preparation of these preferred LRCAs of the present invention may be conducted as follows:

1) Galactosylation of HSA;

2) Maleimide derivatization of the galactosylated HSA; and

3) Attachment of biotin-liver retention component via a sulfhydryl group to the maleimide-derivatized galactosylated HSA. An example of this process is set forth in detail in the Example IV.

The preferred LRCAs of the present invention were evaluated using two criteria:

A) Effectiveness at clearing targeting moiety-streptavidin conjugate; and

B) Biotin release from the LRCA construct.

These criteria were evaluated in comparison to Galactose-HSA-biotin clearing agents previously developed by the assignee of this patent application as set forth in Example V; however, evaluation of LRCAs does not require such a comparison. Thus, LRCAs can be evaluated in comparison to each other or to established performance criteria.

All four preferred constructs cleared targeting moiety-streptavidin conjugate comparably to the previously developed conjugate, galactose$_{30-35}$-human serum albumin-LC-biotin, wherein LC is an aminocaproyl spacer and wherein the number of biotins ranges from 1 to about 6. At low doses of agent, LRCAs appear to release significantly less biotin, and the trend continues at higher doses.

One embodiment of the present invention provides LRCAs having physical properties facilitating use for in vivo complexation and blood clearance of anti-ligand/ligand (e.g., avidin/biotin)-targeting moiety (e.g., antibody) conjugates. These LRCAs are useful in improving the target:blood ratio of targeting moiety-containing conjugate. One application in which target:blood ratio improvement is sought is in solid tumor imaging and therapy.

Other applications of these LRCAs include lesional imaging or therapy involving blood clots and the like, employing antibody or other targeting vehicle-active agent

delivery modalities. For example, an efficacious anti-clotting agent provides rapid target localization and high target:non-target ratio. Active agents administered in pretargeting protocols of the present invention using efficient clearing agents are targeted in the desirable manner and are, therefore, useful in the imaging/therapy of conditions such as pulmonary embolism and deep vein thrombosis.

The present invention provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;

thereafter administering to the recipient a LRCA incorporating a hepatic clearance directing component capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, a liver retention component, a structural component and a binding component; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate.

Clearing agent evaluation experimentation involving galactose- and biotin-derivatized clearing agents is detailed in Example III. The specific clearing agents examined during the Example III experimentation are human serum albumin derivatized with galactose and biotin and a 70,000 dalton molecular weight dextran derivatized with both biotin and galactose. The experimentation showed that proteins and polymers are derivatizable to contain both galactose and biotin and that the resultant derivatized molecule is effective in removing circulating streptavidin-protein conjugate from the serum of the recipient. Biotin loading was varied to determine the effects on both clearing the blood pool of circulating avidin-containing conjugate and the ability to deliver a subsequently administered biotinylated isotope to a target site recognized by the streptavidin-containing conjugate. The effect of relative doses of the administered components with respect to clearing agent efficacy was also examined. Preparation of LRCAs of the present invention is discussed in Example IV below. Experimentation relating to the LRCAs of the present invention is set forth in Example V below.

The present invention provides LRCAs that incorporate ligand derivatives or anti-ligand derivatives, wherein such derivatives exhibit a lower affinity than the native form of the compound, employed in the same construct, for the complementary ligand/anti-ligand pair member (i.e., lower affinity ligands or anti-ligands). In embodiments of the present invention employing a biotin-avidin or biotin-streptavidin ligand/anti-ligand pair, preferred LRCAs incorporate either lower affinity biotin (which exhibits a lower affinity for avidin or streptavidin than native biotin) or lower affinity avidin or a streptavidin (which exhibits a lower affinity for biotin than native avidin or streptavidin).

In two-step pretargeting protocols employing the biotin-avidin or biotin-streptavidin ligand-anti-ligand pair, lower affinity biotin, lower affinity avidin or lower affinity streptavidin may be employed. Exemplary lower affinity biotin molecules, for example, exhibit the following properties: bind to avidin or streptavidin with an affinity less than that of native biotin (10^{-15}); retain specificity for binding to avidin or streptavidin; are non-toxic to mammalian recipients; and the like. Exemplary lower affinity avidin or streptavidin molecules, for example, exhibit the following properties: bind to biotin with an affinity less than native avidin or streptavidin; retain specificity for binding to biotin; are non-toxic to mammalian recipients; and the like.

Exemplary lower affinity biotin molecules include 2'-thiobiotin; 2'-iminobiotin; 1'-N-methoxycarbonyl-biotin; 3'-N-methoxycarbonylbiotin; 1-oxy-biotin; 1-oxy-2'-thiobiotin; 1-oxy-2'-iminobiotin; 1-sulfoxide-biotin; 1-sulfoxide-2'-thiobiotin; 1-sulfoxide-2'-iminobiotin; 1-sulfone-biotin; 1-sulfone-2'-thio-biotin; 1-sulfone-2'-iminobiotin; imidazolidone derivatives such as desthiobiotin (d and dl optical isomers), dl-desthiobiotin methyl ester, dl-desthiobiotinol, D-4-n-hexyl-imidazolidone, L-4-n-hexylimidazolidone, dl-4-n-butyl-imidazolidone, dl-4-n-propylimidazolidone, dl-4-ethyl-imidazolidone, dl-4-methylimidazolidone, imidazolidone, dl-4,5-dimethylimidazolidone, meso-4,5-dimethylimidazolidone, dl-norleucine hydantoin, D-4-n-hexyl-2-thiono-imidazolidine, d-4-n-hexyl-2-imino-imidazolidine and the like; oxazolidone derivatives such as D-4-n-hexyl-oxazolidone, D-5-n-hexyloxazolidone and the like; [5-(3,4-diamino-thiophan-2-yl] pentanoic acid; lipoic acid; 4-hydroxy-azobenzene-2'-carboxylic acid; and the like. Preferred lower affinity biotin molecules

for use in the practice of the present invention are 2'-thiobiotin, desthiobiotin, 1-oxy-biotin, 1-oxy-2'-thiobiotin, 1-sulfoxide-biotin, 1-sulfoxide-2'-thiobiotin, 1-sulfone-biotin, 1-sulfone-2'-thiobiotin, lipoic acid and the like. These exemplary lower affinity biotin molecules may be produced substantially in accordance with known procedures therefor. Incorporation of the exemplary lower affinity biotin molecules into LRCAs proceeds substantially in accordance with procedures described herein in regard to biotin incorporation.

Much has been reported about the binding affinity of different biotin analogs to avidin. Based upon what is known in the art, the ordinary skilled artisan could readily select or use known techniques to ascertain the respective binding affinity of a particular biotin analog to streptavidin, avidin or a derivative thereof.

The present invention further provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;

thereafter administering to the recipient a LRCA incorporating a hepatic clearance directing component capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, a liver retention component and a binding component including a lower affinity complementary member of the ligand-anti-ligand binding pair employed in the first conjugate; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate and, preferably, constitutes a native or high affinity form thereof.

The "targeting moiety" of the present invention binds to a defined target cell population, such as tumor cells. Preferred targeting moieties useful in this regard include antibody and antibody fragments, peptides, and hormones. Proteins corresponding to known cell surface receptors (including low density lipoproteins, transferrin and insulin), fibrinolytic enzymes, anti-HER2, platelet binding proteins such as annexins, and biological response modifiers (including interleukin, interferon, erythropoietin and colony-stimulating factor) are also preferred targeting moieties.

Also, anti-EGF receptor antibodies, which internalize following binding to the receptor and traffic to the nucleus to an extent, are preferred targeting moieties for use in the present invention to facilitate delivery of Auger emitters and nucleus binding drugs to target cell nuclei. Oligonucleotides, e.g., antisense oligonucleotides that are complementary to portions of target cell nucleic acids (DNA or RNA), are also useful as targeting moieties in the practice of the present invention. Oligonucleotides binding to cell surfaces are also useful. Analogs of the above-listed targeting moieties that retain the capacity to bind to a defined target cell population may also be used within the claimed invention. In addition, synthetic targeting moieties may be designed.

Functional equivalents of the aforementioned molecules are also useful as targeting moieties of the present invention. One targeting moiety functional equivalent is a "mimetic" compound, an organic chemical construct designed to mimic the proper configuration and/or orientation for targeting moiety-target cell binding. Another targeting moiety functional equivalent is a short polypeptide designated as a "minimal" polypeptide, constructed using computer-assisted molecular modeling and mutants having altered binding affinity, which minimal polypeptides exhibit the binding affinity of the targeting moiety.

Preferred targeting moieties of the present invention are antibodies (polyclonal or monoclonal), peptides, oligonucleotides or the like. Polyclonal antibodies useful in the practice of the present invention are polyclonal (Vial and Callahan, Univ. Mich. Med. Bull., 20: 284-6, 1956), affinity-purified polyclonal or fragments thereof (Chao et al., Res. Comm. in Chem. Path. & Pharm., 9: 749-61, 1974).

Monoclonal antibodies useful in the practice of the present invention include whole antibody and fragments thereof. Such monoclonal antibodies and fragments are producible in accordance with conventional techniques, such as hybridoma synthesis, recombinant DNA techniques and protein synthesis. Useful monoclonal antibodies and fragments may be derived from any species (including humans) or may be formed as chimeric proteins which employ sequences from more than one species. See, generally, Kohler and Milstein, Nature, 256: 495-97, 1975; Eur. J. Immunol., 6: 511-19, 1976.

Human monoclonal antibodies or "humanized" murine antibody are also useful as targeting moieties in accordance with the present invention. For example, murine monoclonal antibody may be "humanized" by genetically recombining the nucleotide sequence encoding the murine Fv region (i.e., containing the antigen binding sites) or the complementarity determining regions thereof with the nucleotide sequence encoding a human constant domain region and an Fc region, e.g., in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Some murine residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics. Humanized targeting moieties are recognized to decrease the immunoreactivity of the antibody or polypeptide in the host recipient, permitting an increase in the half-life and a reduction in the possibility of adverse immune reactions.

Types of active agents (diagnostic or therapeutic) useful herein include toxins, anti-tumor agents, drugs and radionuclides. Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only the toxin A chain to a targeting protein. However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the toxin A chain-targeting protein conjugate, as compared to the corresponding holotoxin-targeting protein conjugate.

Preferred toxins in this regard include holotoxins, such as abrin, ricin, modeccin, Pseudomonas exotoxin A, Diphtheria toxin, pertussis toxin and Shiga toxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain, modeccin A chain, the enzymatic portion of Pseudomonas exotoxin A, Diphtheria toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin and snake venom peptides. Ribosomal inactivating proteins (RIPs), naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability, are also suitable for use herein. Extremely highly toxic toxins, such as palytoxin and the like, are also contemplated for use in the practice of the present invention.

Preferred drugs suitable for use herein include conventional chemotherapeutics, such as vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 6-thioguanine, cytarabine, cyclophosphamide and cis-platinum, as well as other conventional chemotherapeutics as described in Cancer: Principles and Practice of Oncology, 2d ed., V.T. DeVita, Jr., S. Hellman, S.A. Rosenberg, J.B. Lippincott Co., Philadelphia, PA, 1985, Chapter 14. A particularly preferred drug within the present invention is a trichothecene.

Trichothecenes are drugs produced by soil fungi of the class *Fungi imperfecti* or isolated from *Baccharus megapotamica* (Bamburg, J.R. Proc. Molec. Subcell. Biol. 8:41-110, 1983; Jarvis & Mazzola, Acc. Chem. Res. 15:338-395, 1982). They appear to be the most toxic molecules that contain only carbon, hydrogen and oxygen (Tamm, C. Fortschr. Chem. Org. Naturst. 31:61-117, 1974). They are all reported to act at the level of the ribosome as inhibitors of protein synthesis at the initiation, elongation, or termination phases.

There are two broad classes of trichothecenes: those that have only a central sesquiterpenoid structure and those that have an additional macrocyclic ring (simple and macrocyclic trichothecenes, respectively). The simple trichothecenes may be subdivided into three groups (i.e., Group A, B, and C) as described in U.S. Patent Nos. 4,744,981 and 4,906,452 (incorporated herein by reference). Representative examples of Group A simple trichothecenes include: Scirpene, Roridin C, dihydrotrichothecene, Scirpen-4, 8-diol, Verrucarol, Scirpentriol, T-2 tetraol, pentahydroxyscirpene, 4-deacetylneosolaniol, trichodermin, deacetylcalonectrin, calonectrin, diacetylverrucarol, 4-monoacetoxyscirpenol, 4,15-diacetoxyscirpenol, 7-hydroxydiacetoxyscirpenol, 8-hydroxydiacetoxy-scirpenol (Neosolaniol), 7,8-dihydroxydiacetoxyscirpenol, 7-hydroxy-8-acetyldiacetoxyscirpenol, 8-acetylneosolaniol, NT-1, NT-2, HT-2, T-2, and acetyl T-2 toxin.

Representative examples of Group B simple trichothecenes include: Trichothecolone, Trichothecin, deoxynivalenol, 3-acetyldeoxynivalenol, 5-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol, Nivalenol, 4-acetylnivalenol (Fusarenon-X), 4,15-idacetylnivalenol, 4,7,15-triacetylnivalenol, and tetra-acetylnivalenol. Representative examples of Group C simple trichothecenes include.

Crotocol and Crotocin. Representative macrocyclic trichothecenes include Verrucarin A, Verrucarin B, Verrucarin J (Satratoxin C), Roridin A, Roridin D, Roridin E (Satratoxin D), Roridin H, Satratoxin F, Satratoxin G, Satratoxin H, Vertisporin, Mytoxin A, Mytoxin C, Mytoxin B, Myrotoxin A, Myrotoxin B, Myrotoxin C, Myrotoxin D, Roritoxin A, Roritoxin B, and Roritoxin D. In addition, the general "trichothecene" sesquiterpenoid ring structure is also present in compounds termed "baccharins" isolated from the higher plant *Baccharis megapotamica*, and these are described in the literature, for instance as disclosed by Jarvis et al. (Chemistry of Alleopathy, ACS Symposium Series No. 268: ed. A.C. Thompson, 1984, pp. 149-159).

Experimental drugs, such as mercaptopurine, N-methylformamide, 2-amino-1,3,4-thiadiazole, melphalan, hexamethylmelamine, gallium nitrate, 3% thymidine, dichloromethotrexate, mitoguazone, suramin, bromodeoxyuridine, iododeoxyuridine, semustine, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea, N,N'-hexamethylene-bis-acetamide, azacitidine, dibromodulcitol, Erwinia asparaginase, ifosfamide, 2-mercaptoethane sulfonate, teniposide, taxol, 3-deazauridine, soluble Baker's antifol, homoharringtonine, cyclocytidine, acivicin, ICRF-187, spiromustine, levamisole, chlorozotocin, aziridinyl benzoquinone, spirogermanium, aclarubicin, pentostatin, PALA, carboplatin, amsacrine, caracemide, iproplatin, misonidazole, dihydro-5-azacytidine, 4'-deoxy-doxorubicin, menogaril, triciribine phosphate, fazarabine, tiazofurin, teroxirone, ethiofos, N-(2-hydroxyethyl)-2-nitro-1H-imidazole-1-acetamide, mitoxantrone, acodazole, amonafide, fludarabine phosphate, pibenzimol, didemnin B, merbarone, dihydrolenperone, flavone-8-acetic acid, oxantrazole, ipomeanol, trimetrexate, deoxyspergualin, echinomycin, and dideoxycytidine (see NCI Investigational Drugs, Pharmaceutical Data 1987, NIH Publication No. 88-2141, Revised November 1987) are also preferred.

Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters preferred for therapeutic use. Radionuclides are well-known in the art and include ^{123}I, ^{125}I, ^{130}I, ^{131}I, ^{133}I, ^{135}I, ^{47}Sc, ^{72}As, ^{72}Se, ^{90}Y, ^{88}Y, ^{97}Ru, ^{100}Pd, ^{101m}Rh, ^{119}Sb, ^{128}Ba, ^{197}Hg, ^{211}At, ^{212}Bi, ^{153}Sm,

^{169}Eu, ^{212}Pb, ^{109}Pd, ^{111}In, ^{67}Ga, ^{68}Ga, ^{64}Cu, ^{67}Cu, ^{75}Br, ^{76}Br, ^{77}Br, ^{99m}Tc, ^{11}C, ^{13}N, ^{15}O, ^{166}Ho and ^{18}F. Preferred therapeutic radionuclides include ^{188}Re, ^{186}Re, ^{203}Pb, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{77}Br, ^{211}At, ^{97}Ru, ^{105}Rh, ^{198}Au and ^{199}Ag, ^{166}Ho or ^{177}Lu.

Other anti-tumor agents, e.g., agents active against proliferating cells, are administrable in accordance with the present invention. Exemplary anti-tumor agents include cytokines, such as IL-2, tumor necrosis factor or the like, lectin inflammatory response promoters (selectins), such as L-selectin, E-selectin, P-selectin or the like, and like molecules.

Ligands suitable for use within the present invention include biotin, haptens, lectins, epitopes, dsDNA fragments, enzyme inhibitors and analogs and derivatives thereof. Useful complementary anti-ligands include avidin (for biotin), carbohydrates (for lectins) and antibody, fragments or analogs thereof, including mimetics (for haptens and epitopes) and zinc finger proteins (for dsDNA fragments) and enzymes (for enzyme inhibitors). Preferred ligands and anti-ligands bind to each other with an affinity of at least about k_D 10^9 M. Other useful ligand/anti-ligand systems include S-protein/S-peptide, head activator protein (which binds to itself), cystatin-C/cathepsin B, and the like.

One preferred chelate system for use in the practice of the present invention is based upon a 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetra acetic acid (DOTA) construct. Because DOTA strongly binds Y-90 and other radionuclides, it has been proposed for use in radioimmunotherapy. For therapy, it is very important that the radionuclide be stably bound within the DOTA chelate and that the DOTA chelate be stably attached to an effector, such as a ligand or an anti-ligand.

The strategy for design of preferred DOTA molecules incorporating biotin for use in the practice of embodiments of the present invention involved three primary considerations:

1) in vivo stability (including biotinidase and general peptidase activity resistance), with an initial acceptance criterion of 100% stability for 1 hour;

2) renal excretion; and

3) ease of synthesis.

The same or similar criteria are applicable to alternative binding moieties, such as ligands or anti-ligands, as can be readily ascertained by one of ordinary skill in the art.

The DOTA-biotin conjugates that are preferably employed in the practice of the present invention reflect the implementation of one or more of the following strategies:

1) substitution of the carbon adjacent to the cleavage susceptible amide nitrogen;

2) alkylation of the cleavage susceptible amide nitrogen;

3) substitution of the amide carbonyl with an alkyl amino group;

4) incorporation of D-amino acids as well as analogs or derivatives thereof; or

5) incorporation of thiourea linkages.

DOTA-biotin conjugates in accordance with the present invention are described in published PCT Patent Application No. PCT/US93/05406. A method of preparing a preferred DOTA-biotin embodiment is described in Example II hereof.

The preferred linkers are useful to produce DOTA-biotin or other DOTA-small molecule conjugates having one or more of the following advantages:

- bind avidin or streptavidin with the same or substantially similar affinity as free biotin;

- bind metal M^{+3} ions efficiently and with high kinetic stability;

- are excreted primarily through the kidneys into urine;

- are stable to endogenous enzymatic or chemical degradation (e.g., bodily fluid amidases, peptidases or the like);

- penetrate tissue rapidly and bind to pretargeted avidin or streptavidin; and

- are excreted rapidly with a whole body residence half-life of less than about 5 hours.

One component to be administered in a preferred two-step pretargeting protocol is a targeting moiety-anti-ligand or a targeting moiety-ligand conjugate. Streptavidin-proteinaceous targeting moiety conjugates are preferably prepared as described in Example I below, with the preparation involving the steps of: preparation of SMCC-derivatized streptavidin; preparation of DTT-reduced proteinaceous targeting moiety; conjugation of the two prepared moieties; and purification of the

monosubstituted or disubstituted (with respect to streptavidin) conjugate from crosslinked (antibody-streptavidin-antibody) and aggregate species and unreacted starting materials. The purified fraction is preferably further characterized by one or more of the following techniques: HPLC size exclusion, SDS-PAGE, immunoreactivity, biotin binding capacity and in vivo studies.

LRCAs of the present invention may be administered in single or multiple doses or via continuous infusion. A single dose of a biotin-containing LRCA, for example, produces a rapid decrease in the level of circulating targeting moiety-streptavidin, followed by a small increase in that level, presumably caused, at least in part, by re-equilibration of targeting moiety-streptavidin within the recipient's physiological compartments. A second or additional LRCA doses may then be employed to provide supplemental clearance of targeting moiety-streptavidin. Alternatively, LRCA may be infused intravenously for a time period sufficient to clear targeting moiety-streptavidin in a continuous manner.

The dose of LRCAs of the present invention will depend upon numerous patient-specific and clinical factors, which clinicians are uniquely qualified to assess. In general, the dose of the LRCA to be administered will depend on the dose of the targeting conjugate or other previously administered component to be cleared that is either measured or expected to remain in the serum compartment at the time the LRCA is administered. Alternatively, the dose of the LRCA will depend on the measured or expected level of toxic agent to be cleared. Generally, a single LRCA dose will range from about 200 mg to about 1000 mg, with from about 300 mg to about 700 mg preferred.

One embodiment of the present invention in which rapid acting LRCAs are useful is in the delivery of Auger emitters, such as I-125, I-123, Er-165, Sb-119, Hg-197, Ru-97, Tl-201 and Br-77, or nucleus-binding drugs to target cell nuclei. In these embodiments of the present invention, targeting moieties that localize to internalizing receptors on target cell surfaces are employed to deliver a targeting moiety-containing conjugate (i.e., a targeting moiety-anti-ligand conjugate in the preferred two-step protocol) to the target cell population. Such internalizing receptors include EGF receptors, transferrin receptors, HER2 receptors, IL-2 receptors, other interleukins

and cluster differentiation receptors, somatostatin receptors, other peptide binding receptors and the like.

After the passage of a time period sufficient to achieve localization of the conjugate to target cells, but insufficient to induce internalization of such targeted conjugates by those cells through a receptor-mediated event, a rapidly acting LRCA is administered. In a preferred two-step protocol, an active agent-containing ligand or anti-ligand conjugate, such as a biotin-Auger emitter or a biotin-nucleus acting drug, is administered as soon as the LRCA has been given an opportunity to complex with circulating targeting moiety-containing conjugate, with the time lag between LRCA and active agent administration being less than about 24 hours. In this manner, active agent is readily internalized through target cell receptor-mediated internalization. While circulating Auger emitters are thought to be non-toxic, the rapid, specific targeting afforded by the pretargeting protocols of the present invention increases the potential of shorter half-life Auger emitters, such as I-123, which is available and capable of stable binding.

The invention is further described through presentation of the following examples. These examples are offered by way of illustration, and not by way of limitation.

Example I

Targeting Moiety-Anti-Ligand Conjugate for Two-Step Pretargeting In Vivo

A. Preparation of SMCC-derivatized streptavidin.

31 mg (0.48 mol) streptavidin was dissolved in 9.0 ml PBS to prepare a final solution at 3.5 mg/ml. The pH of the solution was adjusted to 8.5 by addition of 0.9 ml of 0.5 M borate buffer, pH 8.5. A DMSO solution of SMCC (3.5 mg/ml) was prepared, and 477 l (4.8 mol) of this solution was added dropwise to the vortexing protein solution. After 30 minutes of stirring, the solution was purified by G-25 (PD-10, Pharmacia, Picastaway, New Jersey) column chromatography to remove

unreacted or hydrolyzed SMCC. The purified SMCC-derivatized streptavidin was isolated (28 mg, 1.67 mg/ml).

B. Preparation of DTT-reduced NR-LU-10. To 77 mg NR-LU-10 (0.42 mol) in 15.0 ml PBS was added 1.5 ml of 0.5 M borate buffer, pH 8.5. A DTT solution, at 400 mg/ml (165 l) was added to the protein solution. After stirring at room temperature for 30 minutes, the reduced antibody was purified by G-25 size exclusion chromatography. Purified DTT-reduced NR-LU-10 was obtained (74 mg, 2.17 mg/ml).

C. Conjugation of SMCC-streptavidin to DTT- reduced NR-LU-10. DTT-reduced NR-LU-10 (63 mg, 29 ml, 0.42 mol) was diluted with 44.5 ml PBS. The solution of SMCC-streptavidin (28 mg, 17 ml, 0.42 mol) was added rapidly to the stirring solution of NR-LU-10. Total protein concentration in the reaction mixture was 1.0 mg/ml. The progress of the reaction was monitored by HPLC (Zorbax® GF-250, available from MacMod). After approximately 45 minutes, the reaction was quenched by adding solid sodium tetrathionate to a final concentration of 5 mM.

D. Purification of conjugate. For small scale reactions, monosubstituted or disubstituted (with regard to streptavidin) conjugate was obtained using HPLC Zorbax (preparative) size exclusion chromatography. The desired monosubstituted or disubstituted conjugate product eluted at 14.0-14.5 min (3.0 ml/min flow rate), while unreacted NR-LU-10 eluted at 14.5-15 min and unreacted derivatized streptavidin eluted at 19-20 min.

For larger scale conjugation reactions, monosubstituted or disubstituted adduct is isolatable using DEAE ion exchange chromatography. After concentration of the crude conjugate mixture, free streptavidin was removed therefrom by eluting the column with 2.5% xylitol in sodium borate buffer, pH 8.6. The bound unreacted antibody and desired conjugate were then sequentially eluted from the column using an increasing salt gradient in 20 mM diethanolamine adjusted to pH 8.6 with sodium hydroxide.

E. Characterization of Conjugate.

1. HPLC size exclusion was conducted as described above with respect to small scale purification.

2. SDS-PAGE analysis was performed using 5% polyacrylamide gels under non-denaturing conditions. Conjugates to be evaluated were not boiled in sample buffer containing SDS to avoid dissociation of streptavidin into its 15 kD subunits. Two product bands were observed on the gel, which correspond to the mono- and di-substituted conjugates.

3. Immunoreactivity was assessed, for example, by competitive binding ELISA as compared to free antibody. Values obtained were within 10% of those for the free antibody.

4. Biotin binding capacity was assessed, for example, by titrating a known quantity of conjugate with p-[I-125]iodobenzoylbiocytin. Saturation of the biotin binding sites was observed upon addition of 4 equivalences of the labeled biocytin.

5. In vivo studies are useful to characterize the reaction product, which studies include, for example, serum clearance profiles, ability of the conjugate to target antigen-positive tumors, tumor retention of the conjugate over time and the ability of a biotinylated molecule to bind streptavidin conjugate at the tumor. These data facilitate determination that the synthesis resulted in the formation of a 1:1 streptavidin-NR-LU-10 whole antibody conjugate that exhibits blood clearance properties similar to native NR-LU-10 whole antibody, and tumor uptake and retention properties at least equal to native NR-LU-10.

For example, Figure 1 depicts the tumor uptake profile of the NR-LU-10-streptavidin conjugate (Ab/SA, referred to in this example as LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody and a control profile of streptavidin. LU-10-StrAv was radiolabeled on the streptavidin component only, giving a clear indication that LU-10-StrAv localizes to target cells as efficiently as NR-LU-10 whole antibody itself.

Example II

Synthesis of DOTA-Biotin Conjugates

A. Synthesis of Nitro-Benzyl-DOTA.

The synthesis of aminobenzyl-DOTA was conducted substantially in accordance with the procedure of McMurry et al., Bioconjugate Chem., 3: 108-117, 1992. The

critical step in the prior art synthesis is the intermolecular cyclization between disuccinimidyl N-(tert-butoxycarbonyl)iminodiacetate and N-(2-aminoethyl)-4-nitrophenyl alaninamide to prepare 1-(tert-butoxycarbonyl)-5-(4-nitrobenzyl)-3,6,11-trioxo-1,4,7,10-tetraazacyclododecane. In other words, the critical step is the intermolecular cyclization between the bis-NHS ester and the diamine to give the cyclized dodecane. McMurry et al. conducted the cyclization step on a 140 mmol scale, dissolving each of the reagents in 100 ml DMF and adding via a syringe pump over 48 hours to a reaction pot containing 4 liters dioxane.

A 5x scale-up of the McMurry et al. procedure was not practical in terms of reaction volume, addition rate and reaction time. Process chemistry studies revealed that the reaction addition rate could be substantially increased and that the solvent volume could be greatly reduced, while still obtaining a similar yield of the desired cyclization product. Consequently on a 30 mmol scale, each of the reagents was dissolved in 500 ml DMF and added via addition funnel over 27 hours to a reaction pot containing 3 liters dioxane. The addition rate of the method employed involved a 5.18 mmol/hour addition rate and a 0.047 M reaction concentration.

B. Synthesis of an N-methyl-glycine linked conjugate.

The N-methyl glycine-linked DOTA-biotin conjugate was prepared by an analogous method to that used to prepare D-alanine-linked DOTA-biotin conjugates. N-methyl-glycine (trivial name sarcosine, available from Sigma Chemical Co.) was condensed with biotin-NHS ester in DMF and triethylamine to obtain N-methyl glycyl-biotin. N-methyl-glycyl biotin was then activated with EDCI and NHS. The resultant NHS ester was not isolated and was condensed in situ with DOTA-aniline and excess pyridine. The reaction solution was heated at 60°C for 10 minutes and then evaporated. The residue was purified by preparative HPLC to give [(N-methyl-N-biotinyl)-N-glycyl]-aminobenzyl-DOTA.

1. Preparation of (N-methyl)glycyl biotin. DMF (8.0 ml) and triethylamine (0.61 ml, 4.35 mmol) were added to solids N-methyl glycine (182 mg, 2.05 mmol) and N-hydroxy-succinimidyl biotin (500 mg, 1.46 mmol). The mixture was heated for 1 hour in an oil bath at 85°C during which time the solids dissolved producing a clear

and colorless solution. The solvents were then evaporated. The yellow oil residue was acidified with glacial acetic acid, evaporated and chromatographed on a 27 mm column packed with 50 g silica, eluting with 30% MeOH/EtOAc 1% HOAc to give the product as a white solid (383 mg) in 66% yield.

H-NMR (DMSO): 1.18-1.25 (m, 6H, $(CH_2)_3$), 2.15, 2.35 (2 t's, 2H, CH_2CO), 2.75 (m, 2H, SCH_2), 2.80, 3.00 (2 s's, 3H, NCH_3), 3.05-3.15 (m, 1H, SCH), 3.95, 4.05 (2 s's, 2H, CH_2N), 4.15, 4.32 (2 m's, 2H, 2CHN's), 6.35 (s, NH), 6.45 (s, NH).

2. Preparation of [(N-methyl-N-biotinyl)glycyl] aminobenzyl-DOTA. N-hydroxysuccinimide (10 mg, 0.08 mmol) and EDCI (15 mg, 6.08 mmol) were added to a solution of (N-methylglycyl biotin (24 mg, 0.08 mmol) in DMF (1.0 ml). The solution was stirred at 23 C for 64 hours. Pyridine (0.8 ml) and aminobenzyl-DOTA (20mg, 0.04 mmol) were added. The mixture was heated in an oil bath at 63°C for 10 minutes, then stirred at 23 C for 4 hours. The solution was evaporated. The residue was purified by preparative HPLC to give the product as an off white solid (8 mg, 0.01 mmol) in 27% yield.

H-NMR (D_2O): 1.30-1.80 (m, 6H), 2.40, 2.55 (2 t's, 2H, CH_2CO), 2.70-4.2 (complex multiplet), 4.35 (m, CHN), 4.55 (m, CHN), 7.30 (m, 2H, benzene hydrogens), 7.40 (m, 2H, benzene hydrogens).

EXAMPLE III

Clearing Agent Evaluation Experimentation

A. Galactose- and Biotin-Derivatization of Human Serum Albumin (HSA). HSA was evaluated because it exhibits the advantages of being both inexpensive and non-immunogenic. HSA was derivatized with varying levels of biotin (1-about 9 biotins/molecule) via analogous chemistry to that previously described with respect to AO. More specifically, to a solution of HSA available from Sigma Chemical Co. (5-10 mg/ml in PBS) was added 10% v/v 0.5 M sodium borate buffer, pH 8.5, followed by dropwise addition of a DMSO solution of NHS-LC-biotin (Sigma Chemical Co.) to the stirred solution at the desired molar offering (relative molar equivalents of reactants). The final percent DMSO in the reaction mixture should not exceed 5%.

After stirring for 1 hour at room temperature, the reaction was complete. A 90% incorporation efficiency for biotin on HSA was generally observed. As a result, if 3 molar equivalences of the NHS ester of LC-biotin was introduced, about 2.7 biotins per HSA molecule were obtained. Unreacted biotin reagent was removed from the biotin-derivatized HSA using G-25 size exclusion chromatography. Alternatively, the crude material may be directly galactosylated. The same chemistry is applicable for biotinylating non-previously biotinylated dextran.

HSA-biotin was then derivatized with from 12 to 45 galactoses/molecule. Galactose derivatization of the biotinylated HSA was performed according to the procedure of Lee, et al., Biochemistry, 15: 3956, 1976. More specifically, a 0.1 M methanolic solution of cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-D-galactopyranoside was prepared and reacted with a 10% v/v 0.1 M NaOMe in methanol for 12 hours to generate the reactive galactosyl thioimidate. The galactosylation of biotinylated HSA began by initial evaporation of the anhydrous methanol from a 300 fold molar excess of reactive thioimidate. Biotinylated HSA in PBS, buffered with 10% v/v 0.5 M sodium borate, was added to the oily residue. After stirring at room temperature for 2 hours, the mixture was stored at 4°C for 12 hours. The galactosylated HSA-biotin was then purified by G-25 size exclusion chromatography or by buffer exchange to yield the desired product. The same chemistry is exploitable to galactosylating dextran. The incorporation efficiency of galactose on HSA is approximately 10%.

70 micrograms of Galactose-HSA-Biotin (G-HSA-B), with 12-45 galactose residues and 9 biotins, was administered to mice which had been administered 200 micrograms of StrAv-MAb or 200 microliters of PBS 24 hours earlier. Results indicated that G-HSA-B is effective in removing StrAv-MAb from circulation. Also, the pharmacokinetics of G-HSA-B is unperturbed and rapid in the presence or absence of circulating MAb-StrAv.

B. Non-Protein Clearing Agent. A commercially available form of dextran, molecular weight of 70,000 daltons, pre-derivatized with approximately 18 biotins/molecule and having an equivalent number of free primary amines was studied. The primary amine moieties were derivatized with a galactosylating reagent,

substantially in accordance with the procedure therefor described above in the discussion of HSA-based clearing agents, at a level of about 9 galactoses/molecule. The molar equivalence offering ratio of galactose to HSA was about 300:1, with about one-third of the galactose being converted to active form. 40 Micrograms of galactose-dextran-biotin (GAL-DEX-BT) was then injected i.v. into one group of mice which had received 200 micrograms MAb-StrAv conjugate intravenously 24 hours earlier, while 80 micrograms of GAL-DEX-BT was injected into other such mice. GAL-DEX-BT was rapid and efficient at clearing StrAv-MAb conjugate, removing over 66% of circulating conjugate in less than 4 hours after clearing agent administration. An equivalent effect was seen at both clearing agent doses, which correspond to 1.6 (40 micrograms) and 3.2 (80 micrograms) times the stoichiometric amount of circulating StrAv conjugate present.

C. Dose Ranging for G-HSA-B Clearing Agent. Dose ranging studies followed the following basic format:

200 micrograms MAb-StrAv conjugate administered;

24 hours later, clearing agent administered; and

2 hours later, 5.7 micrograms PIP-biocytin administered.

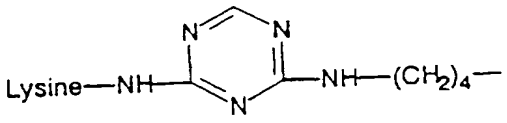
Dose ranging studies were performed with the G-HSA-B clearing agent, starting with a loading of 9 biotins per molecule and 12-45 galactose residues per molecule. Doses of 20, 40, 70 and 120 micrograms were administered 24 hours after a 200 microgram dose of MAb-StrAv conjugate. The clearing agent administrations were followed 2 hours later by administration of 5.7 micrograms of I-131-PIP-biocytin. Tumor uptake and blood retention of PIP-biocytin was examined 44 hours after administration thereof (46 hours after clearing agent administration). The results showed that a nadir in blood retention of PIP-biocytin was achieved by all doses greater than or equal to 40 micrograms of G-HSA-B. A clear, dose-dependent decrease in tumor binding of PIP-biocytin at each increasing dose of G-HSA-B was present, however. Since no dose-dependent effect on the localization of MAb-StrAv conjugate at the tumor was observed, this data was interpreted as being indicative of relatively higher blocking of tumor-associated MAb-StrAv conjugate by the release of biotin from catabolized clearing agent. Similar results to those described earlier for

the asialoorosomucoid clearing agent regarding plots of tumor/blood ratio were found with respect to G-HSA-B, in that an optimal balance between blood clearance and tumor retention occurred around the 40 microgram dose. Because of the relatively large molar amounts of biotin that could be released by this clearing agent at higher doses, studies were undertaken to evaluate the effect of lower levels of biotinylation on the effectiveness of the clearing agent. G-HSA-B, derivatized with either 9, 5 or 2 biotins/molecule, was able to clear MAb-StrAv conjugate from blood at equal protein doses of clearing agent. All levels of biotinylation yielded effective, rapid clearance of MAb-StrAv from blood.

Comparison of these 9-, 5-, and 2-biotin-derivatized clearing agents with a single biotin G-HSA-B clearing agent was carried out in tumored mice, employing a 60 microgram dose of each clearing agent. This experiment showed each clearing agent to be substantially equally effective in blood clearance and tumor retention of MAb-StrAv conjugate 2 hours after clearing agent administration. The G-HSA-B with a single biotin was examined for the ability to reduce binding of a subsequently administered biotinylated small molecule (PIP-biocytin) in blood, while preserving tumor binding of PIP-biocytin to prelocalized MAb-StrAv conjugate. Measured at 44 hours following PIP-biocytin administration, tumor localization of both the MAb-StrAv conjugate and PIP-biocytin was well preserved over a broad dose range of G-HSA-B with one biotin/molecule (90 to 180 micrograms). A progressive decrease in blood retention of PIP-biocytin was achieved by increasing doses of the single biotin G-HSA-B clearing agent, while tumor localization remained essentially constant, indicating that this clearing agent, with a lower level of biotinylation, is preferred. This preference arises because the single biotin G-HSA-B clearing agent is both effective at clearing MAb-StrAv over a broader range of doses (potentially eliminating the need for patient-to-patient titration of optimal dose) and appears to release less competing biotin into the systemic circulation than the same agent having a higher biotin loading level.

Another way in which to decrease the effect of clearing agent-released biotin on active agent-biotin conjugate binding to prelocalized targeting moiety-streptavidin conjugate is to attach the protein or polymer or other primary clearing agent

component to biotin using a retention linker. A retention linker has a chemical structure that is resistant to agents that cleave peptide bonds and, optionally, becomes protonated when localized to a catabolizing space, such as a lysosome. Preferred retention linkers of the present invention are short strings of D-amino acids or small molecules having both of the characteristics set forth above. An exemplary retention linker of the present invention is cyanuric chloride, which may be interposed between an epsilon amino group of a lysine of a proteinaceous primary clearing agent component and an amine moiety of a reduced and chemically altered biotin carboxy moiety (which has been discussed above) to form a compound of the structure set forth below.



When the compound shown above is catabolized in a catabolizing space, the heterocyclic ring becomes protonated. The ring protonation prevents the catabolite from exiting the lysosome. In this manner, biotin catabolites containing the heterocyclic ring are restricted to the site(s) of catabolism and, therefore, do not compete with active-agent-biotin conjugate for prelocalized targeting moiety-streptavidin target sites

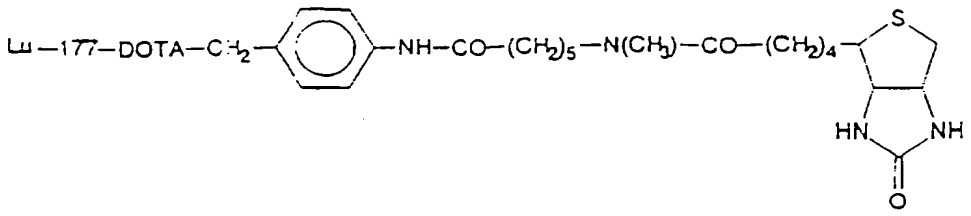
Comparisons of tumor/blood localization of radiolabeled PIP-biocytin observed in the G-HSA-B dose ranging studies showed that optimal tumor to background targeting was achieved over a broad dose range (90 to 180 micrograms), with the results providing the expectation that even larger clearing agent doses would also be effective. Another key result of the dose ranging experimentation is that G-HSA-B with an average of only 1 biotin per molecule is presumably only clearing the MAb-StrAv conjugate via the Ashwell receptor mechanism only, because too few biotins are present to cause cross-linking and aggregation of MAb-StrAv conjugates and clearing agents with such aggregates being cleared by the reticuloendothelial system.

D. Tumor Targeting Evaluation Using G-HSA-B. The protocol for this experiment was as follows:

Time 0: administer 400 micrograms MAb-StrAv conjugate;

Time 24 hours: administer 240 micrograms of G-HSA-B with one biotin and 12-45 galactoses and

Time 26 hours: administer 6 micrograms of



Lu-177 is complexed with the DOTA chelate using known techniques therefor.

Efficient delivery of the Lu-177-DOTA-biotin small molecule was observed, 20-25 % injected dose/gram of tumor. These values are equivalent with the efficiency of the delivery of the MAb-StrAv conjugate. The AUC tumor/AUC blood obtained for this non-optimized clearing agent dose was 300% greater than that achievable by comparable direct MAb-radiolabel administration. Subsequent experimentation has resulted in AUC tumor/AUC blood over 1000% greater than that achievable by comparable conventional MAb-radiolabel administration. In addition, the HSA-based clearing agent is expected to exhibit a low degree of immunogenicity in humans.

EXAMPLE IV

LRCA Preparation

A. Hepatic Clearance Directing Moiety-Structural Component-Reactive Group Construct.

1. Galactosylated HSA. An optimized procedure for the preparation of a $(galactose)_{48}$-HSA-$(maleimide)_2$ construct is set forth below. To a solution of 236 mg of cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-D-galactopyranoside (commercially available from Sigma Chemical Co., St. Louis, Missouri) in 5.85 ml of methanol was added 0.58 ml of 0.1 M NaOMe. The mixture was stirred at 15-25°C for 12-16 hours to afford methanolic 2-imino-2-methoxyethyl-1-thio-galactopyranoside. An aliquot (2.37 ml) of the solution was concentrated. To 100 mg of HSA (commercially available from Sigma Chemical Co.) in 4.08 ml of 20 mM PBS (pH 7.0) was added 0.92 ml of PBS and 0.5 ml of 0.5M borate buffer (pH 8.5). The resultant HSA

solution was then added to the concentrated 2-imino-2-methoxyethyl-1-thiogalactose pyranoside. The mixture was rotated at 15-25°C for 24 hours. The product was purified on 47 ml of G-25 exclusion matrix (commercially available from Pharmacia, Piscastaway, New Jersey) eluting with PBS to afford 10 ml of 9.3 mg protein/ml of product containing 48 galactose residue per HSA. The extent of galactose loading is assayed by STM 05.210.063 Anthrone assay (Viles and Silverman, "Determination of starch and cellulose with anthrone," Anal. Chem., 21: 950-3, 1949).

2. Galactosylated-HSA-Maleimide. To 7.15 ml of the galactose-HSA solution was added 715 microliters of 0.5M borate (pH 8.0). While rotating, 357 microliters of succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) solution in DMSO (10.64 mg/ml) was added. The mixture was stirred for 60 minutes at 15-25°C. The resultant mixture was purified on 175 ml of G-25 size exclusion matrix, eluting with PBS, to afford 35 ml of 2.85 mg/ml of galactosylated HSA with, on average, 2.0 maleimides/HSA. The product was concentrated via Amicon filtration (30,000 MW cutoff), commercially available from Amicon, Beverly, Mass., to a concentration of 6.4 mg/ml (14 ml total). The solution was sterile filtered (using 0.2 micrometer filters), dispensed into cryogenic vials and stored at -70°C.

B. Liver Retention Component-Binding Component Constructs.

B1. As shown in Figure 2, the liver retention component-binding component construct, N-methyl-N-{5'-[methylester tris-(-(D, L)-phosphonoalanyl)-(D)-cystyl]-5-carbamylpentyl}biotinamide (11), may be prepared as described below.

(D, L)-Methyl-2-amino-3-diethylphosphono-propionate (1). To a solution of 3.771 g (22.3 mmole) of (D, L)-2-amino-3-phosphono-propionic acid (Aldrich Chemical, Milwaukee, WI) in 110 ml dry MeOH at -5°C was added dropwise 22.3 ml of $SOCl_2$. The reaction mixture was stirred at room temperature under nitrogen overnight, at which time solvent was removed under vacuum to give a methyl ester intermediate. This intermediate was taken up in 200 ml anhydrous $HC(OEt)_3$ and heated at 125-165°C for 4 hours with constant removal of EtOH via a Vigreux distilling head

(Chem Glass Inc., Vineland, New Jersey). Excess $HC(OEt)_3$ was then removed under vacuum to give a crude intermediate. This crude intermediate was purified by flash column silica gel chromatography (eluting solvent: CH_2Cl_2\acetone\ 2-propanol = 85:10:5) to give 2.395 g of the N-formyl derivative of the desired product. This N-formyl derivative was dissolved in dry MeOH and saturated with HCl gas. To this HCl saturated solution was added 0.6 ml $SOCl_2$. The reaction mixture was stirred at room temperature under N_2 overnight, at which time solvent was removed under vacuum and the resulting residue was co-evaporated once with dry CH_3CN to give 2.364 g of the hydrogen chloride salt of the desired product. (39% yield) ^{1}H-NMR (CD_3OD, δ): 4.20 (m, CH & CH_2O), 3.87 (s, OCH_3), 2.48 (m, CH_2-P), and 1.33 (t, CH_3).

(D, L)-Methyl-N-BOC-2-amino-3-diethylphosphono-propionate (2). To a solution of 1.682 g (6.1 mmole) of (1) in 32 ml dry CH_3CN and 1.643 ml (2 eq) of dry Et_3N was added a solution of 1.392 g (1.05 eq) of BOC anhydride (Aldrich Chemical) in 20 ml dry CH_3CN. The reaction mixture was stirred at room temperature overnight under nitrogen, at which time thin layer chromatography (eluting solvent: CH_2Cl_2\MeOH = 9:1) showed that the reaction was not yet completed. Consequently, to the reaction mixture was added 0.1 g of BOC anhydride and 0.2 ml Et_3N. The reaction mixture was allowed to continue to stir at room temperature for 4 hours, at which time solvent was removed under vacuum to give a crude residue. After the usual work-up in EtOAc (i.e., washed with water, saturated NaCl, dried over Na_2SO_4, filtered and evaporated), the crude product was purified by flash column silica gel chromatography (eluting solvent: EtOAc) to give 1.206 g pure product (2). (58% yield) ^{1}H-NMR ($CDCl_3$, δ): 5.70 (d, 1H, NH), 4.62 & 4.49 (m & m, 1H, CH), 4.10 (m, 4H, CH_2O), 3.77 (s, 3H, OCH_3), 2.33 (m, 2H, CH_2-P), 1.45 (s, 9H, t-butyl) and 1.33 (t, 6H, CH_3).

(D, L)-N-BOC-2-Amino-3-diethylphosphono-propionic acid (3). To a solution of 1.2 g (3.53 mmole) of (2) in 7 ml MeOH was added 7.26 ml (1.02 eq) of 0.5N NaOH. The reaction mixture was heated at reflux temperature for 3 hours. Solvent was

removed under vacuum, and the resulting residue was dissolved in 12 ml water and extracted twice with 10 ml ether. The water layer was adjusted to pH 3 with 6N HCl and extracted with EtOAc. The aqueous layer was then further adjusted to pH 1 with 6N HCl and extracted four more times with EtOAc. The EtOAc extractions were combined, dried over Na_2SO_4, filtered and evaporated to give a crystalline solid (3). (1.094 g; 95% yield) ^{1}H-NMR ($CDCl_3$, δ): 5.74 (d, 1H, NH), 4.56 & 4.42 (m & m, 1H, CH), 4.15 (m, 4H, CH_2O), 2.50 (dd, 2H, CH_2-P), 1.44 (s, 9H, t-butyl) and 1.33 (two sets of t, 6H, CH_3).

N-BOC-(D, L)-Diethylphosphono-alanyl-(D, L)-diethylphosphono-alanyl methyl ester (4). To a solution of 4.75 mg (1.46 mmole) of (3) and 839 mg (1.3 eq) of benzotriazolyloxytris(dimethylamino)phosphonium hexaphosphonate (BOP, commercially available from Aldrich Chemical Company and Chem-Impex International, Wood Dale, Illinois) in 9 ml dry DMF was added simultaneously a solution of 463 mg (1.68 mmole; 1.15 eq) of (1) in 6.3 ml dry DMF and 1.02 ml (4 eq) diisopropylethylamine. The reaction mixture was stirred under nitrogen at room temperature for 2-3 hours, at which time solvent was removed under high vacuum to give a crude residue. This residue was purified by flash column silica gel chromatography (eluting solvent: EtOAc\MeOH = 93:7) to give 673 mg pure (4). (84% yield) ^{1}H-NMR ($CDCl_3$, δ): 7.75 (dd, 1H, NH), 5.74 (bs, 1H, NH), 4.85 & 4.72 (m & m, 1H, CH), 4.44 (m, 1H, CH), 4.10 (m, 8H, CH_2O), 3.76 (s, 3H, OCH_3), 2.35 (m, 4H, CH_2-P), 1.46 (s, 9H, t-butyl) and 1.33 (m, 12H, CH_3).

(D, L)-Diethylphosphono-alanyl-(D, L)-diethylphosphono-alanyl methyl ester (5). To a solution of 876 mg (1.6 mmole) of (4) in 7.2 ml CH_2Cl_2 was added 10.8 ml trifluoroacetic acid (TFA). The reaction mixture was stirred at room temperature for 30 minutes, at which time solvent was removed under vacuum to give a crude residue. This residue was co-evaporated once with dry toluene and twice with dry CH_3CN to give 1.182g of (5) as 2.55 eq TFA salt. ^{1}H-NMR ($CDCl_3$, δ): 8.35 (d, NH), 4.85 (m, CH), 4.60 (m, CH), 4.15 (m, CH_2O), 3.73 (d, OCH_3), 2.43 (m, CH_2-P), and 1.42 (m, CH_3).

N-BOC-(D, L)-Diethylphosphonoalanyl-(D, L)-diethylphosphono-alanyl-(D, L)-diethylphosphono-alanine methyl ester (6). To a solution of 4.56 mg (1.4 mmole) of (3) and 806 mg (1.3 eq) of BOP in 9 ml of dry DMF was added 1.34 ml (5.5 eq) of diisopropylethylamine and a solution of 1.41 mmole of (5) in 6 ml dry DMF. The reaction mixture was stirred at room temperature under nitrogen for 4 hours. Solvent was removed under high vacuum, and the resulting residue was dissolved in 200 ml EtOAc. The EtOAc layer was washed twice with water, and the water layer was back washed four times with EtOAc. The EtOAc layers were combined and washed with saturated NaCl, dried over Na_2SO_4, filtered and evaporated to give a crude product. This crude product was purified by flash column silica gel chromatography (eluting solvent: CH_2Cl_2\acetone\MeOH = 80:15:5) to give 762 mg product (6). (72% yield) ^{1}H-NMR ($CDCl_3$, δ): 8.28 (m, 1H, NH), 7.83 (m, 1H, NH), 5.90 (m, 1H, NH), 4.90-4.30 (m, 3H, CH), 4.10 (m, 12H, CH_2O), 3.70 (d, 3H, OCH_3), 2.38 (m, 6H, CH_2-P), 1.46 & 1.44 (2s, 9H, t-butyl) and 1.30 (m, 18H, CH_3).

(D, L)-Diethylphosphono-alanyl-(D, L)-diethylphosphono-alanyl-(D, L)-diethyl-phosphono-alanine methyl ester (7). To a solution of 753 mg (1 mmole) of (6) in 5 ml CH_2Cl_2 was added 7 ml TFA. The reaction mixture was stirred at room temperature for 30 minutes, at which time solvent was removed under vacuum to give a crude residue. This residue was co-evaporated twice with dry CH_3CN to give 1.095g gummy (7) as 3 eq of TFA salt. ^{1}H-NMR ($CDCl_3$, δ): 9.17, 8.92, 8.11, 8.00, 7.83 and 7.55 (NH), 4.90-4.35 (m, CH), 4.10 (m, CH_2O), 3.73 (d, OCH_3), 2.45 (m, CH_2-P), and 1.30 (m, CH_3).

N-Methyl-N-(5-(D)-S-tritylcystyl-5-carbamylpentyl)biotinamide (8). N-methyl-N-(5-NHS-carbonyl-pentyl)biotinamide is prepared from biotin (Sigma Chemical Company, St. Louis, Missouri) by the following process: First is BOP-mediated coupling of biotin and N-methyl-epsilon-aminocaproate methylester in DMF and DIEA at room temperature for 3 hours; followed by base hydrolysis (2 eq NaOH in MeOH, room temperature, 14 hours) of the methylester group of the corresponding intermediate, and finally treatment of the obtained N-methyl-N-(5-hydroxycarbonylpentyl)-

biotinamide with N-hydroxysuccinimide in the presence of DCC in DMF at room temperature overnight. To a solution of 2.46 g (5.25 mmole) of N-methyl-N-(5-NHS-carbonyl-pentyl)biotinamide in 2.5 ml dry DMF was added a suspension of 1.818 g (5 mmole) of (D)-S-trityl cysteine (Bachem Bioscience, Inc., King of Prussia, Pennsylvania) and 1.74 ml di-isopropylethylamine (DIEA) (2 eq) in 30 ml of dry DMF. The reaction mixture was stirred at room temperature overnight. Solvent was removed under high vacuum to one-fourth of its original volume. The resulting solution was added dropwise to a stirred 600 ml ether solution to cause precipitation. The precipitate was collected by filtration and partitioned in 70 ml water (pH adjusted to 2 with 0.1N HCl) and 80 ml EtOAc. The water layer was extracted 5 more times with 80 ml EtOAc. The EtOAc layers were combined, brined and dried over with $MgSO_4$ and filtered. The $MgSO_4$ was extensively washed with EtOAc to get all of the product out. The EtOAc filtrate and washes were combined and evaporated to one-third of original volume. Precipitate formed and was collected by filtration to give 2.601 g of off-white solid as the desired product (8). Additional 554 mg of product was obtained after silica gel flash column purification (eluting solvent: CH_2Cl_2\MeOH\HOAc = 84:15:1) of the concentrated residue from the EtOAc filtrate. (88% yield) ^{1}H-NMR ($CDCl_3$, δ): 7.30 (m, 15H, ArH), 6.96, 6.43, 6.01 & 5.10 (NH, 3H), 4.60, 4.48 & 4.30 (3m, 3H, CH), 3,33 (m, 1H, CHS), 3.15 (m, 2H, CH_2N), 3.00-2.65 (m, 4H, CH_2S), 2.90 & 2.85 (2s, 3H, CH_3N), 2.25 (m, 4H, CH_2CO) and 1.80-1.20 (m, 12H, CH_2).

N-Methyl-N-{5-[methylester tris-(D, L)-diethylphosphono-alanyl)-(D)-S-tritvl-cystyl]-5-carbamylpentyl}biotinamide (9). To a solution of 751 mg (1.05 mmole) of (8) and 575 mg (1.3 eq) of BOP in 6 ml dry DMF was added a solution of 1 mmole of (7) in 5 ml dry DMF and 0.87 ml (5 eq) of DIEA. The reaction mixture was stirred at room temperature under nitrogen for 3-4 hours. Solvent was removed under high vacuum, and the resulting residue was worked up in CH_2Cl_2 in the usual manner described above for EtOAc to give a crude product. This crude product was purified by flash column silica gel chromatography (eluting solvent: CH_2Cl_2\MeOH = 95:5; then 90:10; and then 87.5:12.5) to give 1.065 g pure product (9). (79% yield) ^{1}H-

NMR ($CDCl_3$-D_2O, δ): 7.29 (m, 15H, ArH), 4.90-4.20 (m, 5H, CH), 4.02 (m, 12H, CH_2O), 3.70 (br s, 3H, OCH_3), 3.20 (m, 3H, CH_2N & CHS), 2.98-2.50 (m, 7H, CH_3N & CH_2S), 2.50-2.12 (m, 10H, CH_2P & CH_2CO) and 1.85-1.17 (m, 30H, CH_3 & CH_2). ^{31}P-NMR ($CDCl_3$, ppm): 28.74, 27.95, 27.88 & 26.94.

N-Methyl-N-{5-[methylester tris-(-(D, L)-phosphono-alanyl)-(D)-S-trityl-cystyl]-5-carbamylpentyl}biotinamide (10). To a 25 ml round bottom flask charged with 135 mg (0.1 mmole) of (9) was added 7.5 ml of 10% (by volume) trimethylbromosilane (Aldrich Chemical) in dry CH_2Cl_2. The reaction mixture was stirred at room temperature under nitrogen for 4 hours. Solvent was removed, and the resulting residue was treated with 6 ml of 0.05 N triethylammonium bicarbonate (prepared by bulling CO_2 gas to 0.05N triethylamine) (pH 7.1) and 6 ml CH_3CN. The mixture was stirred at room temperature for 15 minutes, at which time solvent was removed, and the resulting residue was dissolved in 20 ml of water and lyophilized. The lyophilization was repeated again to give 171 mg of crude product (10). ^{1}H-NMR (CD_3OD, δ): 7.30 (m, ArH), 4.70-4.25 (m, CH), 3.70 (ms, OCH_3), 3.30 (m, CH_2N & CHS), 3.20 (q, triethylammonium) 3.05-2.55 (m, CH_3N & CH_2S), 2.30 (m, CH_2P & CH_2CO), 1.60 (m, CH_2) and 1.30 (t, triethylammonium). Mass spect. (FAB$^-$) (M/Z): 1183 $[M-H]^-$ and 940 $[M\text{-trityl-H}]^-$. This crude product was used directly in the following reaction without further purification.

N-Methyl-N-{5-[methylester tris-(-(D, L)-phosphono-alanyl)-(D)-cystyl]-5-carbamylpentyl}biotinamide (11). A solution of 100 mg of (10) in 2.38 ml TFA, 0.1 ml anisole and 25 µl ethanedithiol was stirred at room temperature under argon for 2 hours. The reaction mixture was then precipitated into 40 ml ice-cold tert-butyl methylether (deoxygenated with He at 0°C for 10 minutes) in a 50 ml glass centrifuge tube. This tube was capped and centrifuged at 2,000 g for 10 minutes. The supernatant was removed by gentle aspiration. The precipitate was resuspended in 30 ml of deoxygenated tert-butylmethylether, centrifuged again at 2,000 g for 10 minutes, and then supernatant was removed. This procedure was repeated three more times, and the final precipitate was dried under high vacuum for 2 hours to give a

crude (11). ^{1}H-NMR (D_2O, δ): 4.75-4.40 (m, CH), 3.78 (br s, OCH_3), 3.36 (m, CH_2N & CHS), 3.20 (q, triethylammonium TFA salt) 3.08 & 2.90 (2s, CH_3N), 3.04-2.75 (ms, CH_2SH), 2.33 (m, CH_2P & CH_2CO), 1.60 (m, CH_2) and 1.28 (t, triethylammonium TFA salt). Mass spect. (FAB$^-$) (M/Z): 941 [M]$^-$. This crude product was used directly in the conjugation reaction with galactose-derivatized HSA.

B2. As shown in Figure 3, the liver retention component-binding component construct, N-methyl-N-[5-(triglutamylcysteine)-5-carbamylpentyl]-biotinamide, may be prepared as described below.

(D)-N-Fmoc-O-t-butylglutamyl-(D)-S-tritylcytseine (12). To a solution of 2.367 g (4 mmole of (D)-N-Fmoc-γ-O-t-butylglutamate pentafluorophenol ester (Bachem Bioscience, Inc.) in 20 ml dry DMF was added a solution of 1.454 g (4 mmole) of (D)-S-tritylcysteine (Bachem Bioscience, Inc.) and 1.393 ml (2 eq) of DIEA in 20 ml of dry DMF. The reaction mixture was stirred at room temperature under nitrogen overnight, at which time solvent was removed under high vacuum to give a crude residue. This residue was dissolved in EtOAc and washed with 0.1N citric acid (twice), brined and dried over with Na_2SO_4, filtered and evaporated to give a crude product. This crude product was purified by flash column silica gel chromatography (eluting first with EtOAc\CH_2Cl_2 = 9:1 and then with EtOAc\MeOH = 85:15) to give 2.266 g of desired product (12). (74% yield). ^{1}H-NMR ($CDCl_3$, δ): 7.75-7.05 (m, ArH), 6.85 & 6.00 (2br s, NH), 4.20 (m, CH & CH_2O), 2.65 (m, CH_2S), 2.35 (m, CH_2CO & COOH), 1.95 (m, CH_2) and 1.40 (s, t-butyl).

(D)-O-t-butylglutamyl-(D)-S-tritylcytseine (13). A solution of 3.1 g (4.02 mmole) of (12) in 12 ml piperidine and 48 ml DMF was stirred at room temperature for 1 hour. Solvent was removed under high vacuum to give a gummy residue. This residue was solidified while treated with hexane. Hexane was removed by filtration, and the filter was washed six times with hexane to remove the piperidine-Fmoc adduct. The hexane washed solid was purified by flash column reverse-phase C-18

chromatography (eluting solvent: MeOH:water = 65:35 and then 70:30) to give 2.163 g product (13) as the piperidinium salt form. This product was dissolved in 30 ml MeOH and treated with 3.6 ml of 1N NaOH (1 eq). Solvent was removed under vacuum, and the resulting residue was dissolved in 5 ml ether. The ether solution was precipitated into 700 ml stirring hexane. The precipitate was collected by filtration, washed three times with hexane and dried under vacuum to give 1.911 g product (13) as the sodium salt form. (81%) ^{1}H-NMR (CD_3OD, δ): 7.30 (m, 15H, ArH), 4.35 (m, 1H, CH), 3.32 (m, 1H, CH), 2.60 (m, 2H, CH_2S), 2.36 (t, 2H, CH_2CO), 1.90 (m, 2H, CH_2) and 1.39 (s, 9H, t-butyl).

(D)-N-Fmoc-O-t-butylglutamyl-(D)-O-t-butylglutamic acid (14). To a suspension of 2.42 g (4.09 mmole) of (D)-N-Fmoc-γ-O-t-butylglutamate pentafluorophenol ester and 873 mg (1.05 eq of (D)-γ-O-t-butylglutamic acid (Bachem Bioscience, Inc.) In 50 ml dry DMF was added 1.493 ml (2.1 eq) of DIEA. The reaction mixture was stirred at room temperature under nitrogen overnight. Solvent was removed under high vacuum, and the resulting residue was dissolved in EtOAc. The EtOAc layer was washed with 0.1N citric acid twice, water once, brined, dried over Na_2SO_4, filtered and evaporated to give a crude product. This crude product was purified by flash column reverse-phase C-18 chromatography (eluting solvent MeOH\water = 50:50; then 55:45; then 60:40; then 65:35; then 70:30; then 75:25; then 80:20; and then 85:15) to give 2.163 g of product (14). ^{1}H-NMR ($CDCl_3$-D_2O, δ): 7.86, 7.59 & 7.35 (d, d & m, 8H, ArH), 4.53 (m, 1H, CH), 4.40-4.16 (m, 4H, OCH_2 & CH), 2.38 (m, 4H, CH_2CO), 2.05 (m, 4H, CH_2) and 1.45 & 1.42 (2s, 18H, t-butyl).

(D)-N-Fmoc-O-t-butylglutamyl-(D)-O-t-butylglutamate NHS ester (15). To a solution of 2.163 (3.54 mmole) of (14) and 0.428 g (1.05 eq) of N-hydroxysuccinimide in 35 ml dry dioxane was added 803 mg DCC, 1,3-dicyclohexyl-carbodiimide (1.1 eq). The reaction mixture was stirred at room temperature overnight. DCU, 1,3-dicyclohexylurea, was removed by filtration, and the filtrate was evaporated under reduced pressure to give 2.9 g crude (15) in a quantitative yield. ^{1}H-NMR ($CDCl_3$, δ): 7.76, 7.60 & 7.35 (d, d & m, ArH), 5.87 (br d, NH), 4.91 (m,

CH), 4.40-4.15 (m, OCH_2 & CH), 2.83 (s, NHS ester), 2.52-1.80 (m, CH_2CO & CH_2) and 1.46 & 1.44 (2s, t-butyl).

(D)-N-Fmoc-O-t-butylglutamyl-(D)-O-t-butylglutamyl-(D)-O-t-butylglutamyl-(D)-S-tritylcysteine (16). To a solution of 1.358 g (1.66 mmole) of (15) in 7 ml dry DMF was added a solution of 0.9 g (1.58 mmole) of (13) and 275 ml (1 eq) of DIEA in 8 ml dry DMF. The reaction mixture was stirred at room temperature overnight. Solvent was removed under high vacuum, and the resulting residue was dissolved in 200 ml CH_2Cl_2. The CH_2Cl_2 layer was washed twice with 0.1N citric acid, once with water, brined, dried over with Na_2SO_4, filtered and evaporated to give a crude product. This crude product was purified by flash column reverse-phase C-18 chromatography (eluting solvent MeOH\water = 75:25; then 80:20; then 85:15; and then 90:10) to give 587 mg of product (16). (33% yield) ^{1}H-NMR ($CDCl_3$-D_2O, δ): 7.76, 7.51 & 7.28 (d, d & m, 23H, ArH), 4.43-4.08 (m, 6H, OCH_2 & CH), 3.93 (m, 1H, CH), 2.75 (m, 2H, CH_2S), 2.50-1.80 (m, 12H, CH_2CO & CH_2) and 1.46, 1.41 & 1.36 (3s, 27H, t-butyl).

(D)-O-t-butylglutamyl-(D)-O-t-butylglutamyl-(D)-O-t-butylglutamyl-(D)-S-tritylcysteine (17). A solution of 0.58 g (0.51 mmole) of (16) in 1.6 ml piperidine and 6.4 ml DMF was stirred at room temperature for 30 minutes. Solvent was removed under high vacuum, and the resulting residue was dissolved in a minimum amount of EtOAc and then precipitated into hexane. The precipitate was filtered and washed several times with hexane and then vacuum dried to give 449 mg of crude product as the piperidinium salt. This material was dissolved in 40 ml MeOH and treated with 0.76 ml of 1N HCl. Solvent was removed under vacuum, and the resulting residue was dissolved in a minimum amount of MeOH and precipitated into 420 ml of stirring water. The amorphous precipitate was filtered and dried under vacuum to give 396 mg of product (17) as the HCl salt form. ^{1}H-NMR (CD_3OD, δ): 7.28 (m, 15H, ArH), 4.42 (m, 2H, CH), 4.26 (t, 1H, CH), 3.87 (t, 1H, CH), 2.60 (d, 2H, CH_2S), 2.36 (m, 6H, CH_2CO), 2.02 (m, 6H, CH_2) and 1.44, 1.43 & 1.39 (3s, 27H, t-butyl).

N-Methyl-N-{5-[tri-(D)-O-t-butylglutamyl)-(D)-S-tritylcysteine]-5-carbamylpentyl}-biotinamide (18). To a solution of 269 mg (0.27 mmole) of (17) in 3 ml dry DMF was added 113 mg (5 eq) of $NaHCO_3$ followed by addition of a solution of 1.42 mg (1.1 eq) of N-methyl-N-(5-NHS-carbonylpentyl)-biotinamide, prepared as described above in synthesis B1, in 1.5 ml dry DMF. The reaction mixture was stirred at room temperature under nitrogen for 5 hours. The mixture was filtered, and the filtrate was concentrated under reduced pressure to give a crude product. This crude product was purified by flash column reverse-phase C-18 chromatography (eluting solvent MeOH\water = 70:30; then 75:25; then 80:20; then 85:15; and then 90:10) to give 260 mg of product (18). (76% yield) ^{1}H-NMR (CD_3OD, δ): 7.28 (m, 15H, ArH), 4.50-4.20 (m, 6H, CH), 3.35 (m, 2H, CH_2N), 3.18 (m, 1H, CHS), 3.01 & 2.88 (2s, 3H, CH_3N), 2.90 & 2.14 (m & m, 4H, CH_2S), 2.31 (m, 10H, CH_2CO), 2.00 (m, 6H, CH_2) and 1.80-1.25 (m and 3s, 39H, CH_2 & t-butyl).

N-Methyl-N-{5-[tri-(glutamyl)cysteine]-5-carbamylpentyl}-biotinamide (19). A solution of 50 mg (39 μmole) of (18) in 0.95 ml TFA, 40 μl anisole and 10 μl ethanediol was stirred at room temperature for 3 hours. The reaction mixture was precipitated into 30 ml ice-cold deoxygenated tert-butyl methylether in a 50 ml glass centrifuge tube. This tube was capped and centrifuged at 2,000 g for 10 minutes. The supernatant was removed by gentle aspiration. The precipitate was resuspended in 30 ml of deoxygenated tert-butylmethylether and centrifuged again at 2,000 g for 10 minutes, and then supernatant was removed. This procedure was repeated three more times, and the final precipitate was vacuum dried for 30 minutes to give 21 mg of product (19). ^{1}H-NMR (CD_3OD, δ): 4.62-4.26 (m, CH), 3.36 (m, CH_2N), 3.20 (m, CHS), 3.03 & 2.90 (2s, CH_3N), 2.94 (m, CH_2S), 2.69 (d, CH_2S), 2.40 (m, CH_2CO), 2.30-1.85 (m, CH_2) and 1.80-1.25 (m, CH_2). Mass spect. (FAB$^-$) (M/Z): $[M-H]^-$ at 860.

C. LRCA Construct.

The galactose-HSA-maleimide construct is conjugated to the sulfhydryl bearing liver retention-biotin constructs under the following conditions.

Conjugation of (11) with Galactose and Maleimide-Derivatized HSA In a vortexing 5 ml test tube containing 11.5 mg of galactose and maleimide-derivatized HSA described in Section A of this Example in 1.8 ml sterile water and 0.18 ml of 0.2M phosphate buffer (pH 6.5) was added dropwise a solution of 3.7 mg (15 eq of free thiol) of (11) in 380 microliters of sterile water. The reaction mixture was gently shaken at room temperature for 90 minutes. The mixture was transferred into a centricon tube (30K molecular weight cutoff) and centrifuged at 5000 RPM for 10-12 minutes to reduce the mixture volume to 1.2 ml. The mixture plus 100 microliters rinsing was purified by PD-10 Column (Pharmacia Biotech, Inc., Piscataway, New Jersey) eluting with 6 ml PBS. The protein containing fractions were pooled, and the total volume was reduced to 1.12 ml by means of centrifugation in a centricon tube. This 1.12 ml biotin-HSA conjugate solution plus 100 microliters rinsing were transferred to a dialysis cassette (Pierce, Rockford, Illinois) and dialyzed against 1.5L PBS at 4°C for 1 day, at which time fresh PBS (1.5L) was used, and the dialysis was allowed to be continued for 2 days. The dialyzed biotin-HSA solution was sterile filtered and ready to be used as a LRCA. The final protein concentration was determined by UV absorption at 280 nm was 7.81 mg/ml. The molar substitution ratio for biotin/HSA was 2.13 as determined by HABA assay (Green et al., Biochem. J., 94: 236, 1965).

Conjugation of (19) with Galactose and Maleimide-Derivatized HSA. In a vortexing 5 ml test tube containing 11.5 mg of galactose and maleimide-derivatized HSA described in Section A of this Example in 1.8 ml sterile water and 0.18 ml of 0.2M phosphate buffer (pH 6.5) was added dropwise a solution of 3.0 mg (15 eq of free thiol) of (19) in 0.585 ml sterile water and 15 microliters DMSO). The reaction mixture was gently shaken at room temperature for 90 minutes. The mixture was transferred into a centricon tube (30K molecular weight cutoff) and centrifuged at 5000 RPM for 10-12 minutes to reduce the mixture volume to 1.2 ml. The mixture plus 100 microliters rinsing was purified by PD-10 Column (Pharmacia Biotech, Inc., Piscataway, New Jersey) eluting with 6 ml PBS. The protein containing fractions were pooled, and the total volume was reduced to 1.1 ml by means of centrifugation

in a centricon tube. This 1.1 ml biotin-HSA conjugate solution plus 100 microliters rinsing were transferred to a dialysis cassette (Pierce, Rockford, Illinois) and dialyzed against 1.5L PBS at 4°C for 1 day, at which time fresh PBS (1.5L) was used, and the dialysis was allowed to be continued for 2 days. The dialyzed biotin-HSA solution was sterile filtered and ready to be used as a LRCA. The final protein concentration was determined by UV absorption at 280 nm was 8.74 mg/ml. The molar substitution ratio for biotin/HSA was 1.39 as determined by HABA assay (Green et al., Biochem. J., 94: 236, 1965).

EXAMPLE V

LRCA Evaluation

All LRCAs were evaluated in terms of two criteria,

1) Effectiveness at clearing targeting moiety-streptavidin conjugate; and

2) Biotin release from the LRCA construct.

These criteria were evaluated in comparison to a control compound, a galactose-HSA-biotin clearing agent previously developed by the assignee of this patent application, galactose$_{30-35}$-human serum albumin-LC-biotin, wherein LC is an aminocaproyl spacer and wherein the number of biotins ranges from 1 to about 6.

Criteria 1 was evaluated by administering I-125-labeled NR-LU-10-streptavidin conjugate, administering the LRCA 24 hours later, and counting residual conjugate in the blood at a later time point. The animal model employed for this evaluation was female Balb/c mice (20-25 g) injected i.v. with 400 μg I-125-NR-LU-10-streptavidin conjugate prepared substantially as described above. Each group of experimental animals consisted of 3 mice. Blood was sampled serially from the retro-orbital sinus (10 μL x 2) at 1, 4 and 24 hours. At 24 hours, LRCA or the control compound was injected i.v. at total protein doses of either 220 or 1100 μg in each animal. Blood was then sampled 1, 2, 4 and 6 hours post-LRCA administration.

Criteria 2 was evaluated by indirect means. The biotin of the LRCAs was not radiolabeled. Biotin release would be expected to compromise or fill biotin-binding sites of residual serum compartment NR-LU-10-streptavidin conjugate. Thus,

assessment of biotin release from the LRCA was indirectly determined by measuring the amount of In-111-DOTA-biotin, prepared substantially in accordance with Example III hereof, that bound to the residual serum conjugate. Such residual serum conjugate, exposed to LRCAs releasing less biotin-containing metabolites, should exhibit minimal loss of capacity for binding radiolabeled biotin. Criteria 2 was evaluated using the animal model and experimental procedure described for Criteria 1 evaluation, except that 4 hours after LRCA administration, the animals received 1.0 or 15.0 μg of In-111-DOTA-biotin, prepared substantially in accordance with procedures described herein. A blood sample 2 hours after administration of In-111 was used to assess residual biotin-binding capacity of circulating I-125-NR-LU-10-streptavidin conjugate.

Constructs 1 and 2, HSA(d-lys)$_3$BT, HSA(d-lys-gal)$_3$BT were compared to the control compound made in accordance with the procedure set forth in Example IV above, with regard to criteria 1 at clearing agent doses of 220 micrograms and 1100 micrograms. A dose of 220 micrograms has been found to be an optimal dose of the comparison clearing agent in a tumored mouse model to clear 400 micrograms of targeting conjugate. The 1100 microgram dose is 5x the optimal dose. At 200 micrograms, all three compounds cleared conjugate to 4.8-5.6% ID 6 hours after clearing agent administration. At 1100 micrograms, all three compounds cleared conjugate to 2.6-2.9 ID 6 hours following clearing agent administration. Constructs 1 and 2 and the reference compound cleared monoclonal antibody-streptavidin conjugate comparably.

Constructs 1 and 2 were then compared to the reference compound on the basis of criteria 2. The quantity of In-111-DOTA-biotin bound to residual conjugate was measured 2 hours after administration thereof (8 hours after clearing agent administration). Both the 200 microgram and 1100 microgram doses were tested. At the low dose, significantly less blockage of conjugate in the serum (higher In-111 uptake) was observed for constructs 1 and 2 in comparison to the reference compound. The In-111 (serum concentration of In-111-DOTA-biotin)/I-125 (serum concentration of monoclonal antibody-streptavidin conjugate) ratio was 2.66 for construct 1, 2.73 for construct 2 and 0.71 for the reference compound. This trend

was also observed at the high dose, with In-111/I-125 ratios of 0.20 for construct 1, 0.25 for construct 2 and 0.05 for the reference compound.

Construct 3, HSA(d-glu)$_3$BT was analogously evaluated. With respect to criteria 1, serum conjugate concentrations were calculated as %ID/g, rather than %ID as set forth above for the evaluation of constructs 1 and 2. At the 220 microgram clearing agent dose, construct 3 and the reference compound cleared conjugate to 4.96 and 3.41 %ID/g, respectively, 6 hours after clearing agent administration. At the 1100 microgram dose, construct 3 and the reference compound cleared conjugate to 1.19 and 2.02 %ID/g, respectively, 6 hours after clearing agent administration. Construct 3 and the reference compound cleared monoclonal antibody-streptavidin conjugate comparably.

With regard to criteria 2, the comparison at low clearing agent dose showed that significantly less blockage of conjugate in the serum (higher In-111 uptake) occurred when construct 3 was used rather than the reference compound (In-111/I-125 ration of 5.6 for construct 3 and 0.96 for the reference compound). This trend was also observed at the high dose, with In-111/I-125 ratios of 0.83 for construct 3 and 0.18 for the reference compound.

Construct 4, HSA(phos)$_3$BT was evaluated in a tumored mouse model with regard to criteria 2. Criteria 1 was evaluated also, but only at a single time point. At that time point, blood clearance of NR-LU-10-streptavidin was roughly equivalent when construct 4 was compared to the control compound.

In a tumored animal model, compromise of tumor-associated monoclonal antibody-streptavidin conjugate by biotin released from a clearing agent, such as construct 4 or the control compound, can be measured directly, rather than by inference from serum data. As in prior studies, mice were injected with 400 micrograms of conjugate, followed 24 hours later by 200 micrograms (low dose) or 1100 micrograms (high dose) of clearing agent (LRCA or reference compound), followed 4 hours later by 15 micrograms of In-111-DOTA-biotin. Animals were sacrificed 2 hours later. Tumor uptake of In-111-DOTA-biotin was superior at both low and high clearing agent doses (8.89 and 5.01 %ID/g LRCA verses 8.04 and 0.68 %ID/g control).

Targeting of monoclonal antibody-streptavidin to tumor was relatively consistent for all groups of animals at approximately 250 pmol/g. Use of high doses of the reference compound results in significant compromise of ligand binding capacity relative to a low dose offering thereof (1492 pmol/g versus 126 pmol/g). Conversely, construct 4 exhibited only a modest decrease in ligand binding capacity at the tumor in going from low to high LRCA dose (1648 pmol/g versus 929 pmol/g). Thus, much greater dosing latitude appears to be possible with the use of construct 4.

Kits containing one or more of the components described above are also contemplated. For instance, LRCAs may be provided in a sterile container for use in pretargeting procedures. Alternatively, such a LRCA may be vialed in a non-sterile condition for use as a research reagent.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WHAT IS CLAIMED IS:

1. A liver retention clearing agent comprising:

(a) a structural component;

(b) a hepatic clearance directing component directly or indirectly associated with the structural component, wherein the hepatic clearance directing component is composed of a plurality of sugar residues recognized by a hepatocyte receptor;

(c) one or more binding components directly or indirectly associated with the structural component, wherein the binding components are capable of *in vivo* association with a molecule to be cleared; and

(d) one or more liver retention components directly or indirectly associated with the structural component or the binding components or both, such that metabolic processing by hepatocytes of liver retention clearing agent preferentially results in metabolites that contain both a binding component and a liver retention component.

2. A liver retention clearing agent of Claim 1 wherein at least one liver retention component is resistant to peptidase cleavage.

3. A liver retention clearing agent of Claim 2 wherein the liver retention component incorporates at least 3 amino acids of unnatural (D) configuration or unnatural amino acids.

4. A liver retention clearing agent of Claim 3 wherein the liver retention component incorporates from about 3 to about 6 amino acids of unnatural (D) configuration or unnatural amino acids.

5. A liver retention clearing agent of Claim 2 wherein the binding component is bound to either the structural backbone or to the liver retention component via a stable tertiary amide bond.

6. A liver retention clearing agent of Claim 1 wherein the liver retention component is preferentially retained within hepatocyte cytoplasm or a subcellular compartment therein.

7. A liver retention clearing agent of Claim 6 wherein the liver retention component is a DOTA macrocycle.

8. A liver retention clearing agent of Claim 6 wherein the liver retention component incorporates at least three positively charged amino acids of unnatural (D) configuration, at least three negatively charged amino acids of unnatural (D) configuration or at least three neutral charge, hydrophilic amino acids of unnatural (D) configuration.

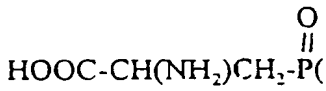
9. A liver retention clearing agent of Claim 6 wherein the liver retention component incorporates at least three positively charged amino acids of natural (L) configuration, at least three negatively charged amino acids of natural (L) configuration or at least three neutral charge, hydrophilic amino acids of natural (L) configuration and wherein at least one stabilized tertiary amide bond is incorporated in the liver retention component or the binding component or in a linker positioned therebetween.

10. A liver retention clearing agent of Claim 8 or Claim 9 wherein the polyamino acids is polylysine, polyglutamic acid, polyhistidine, polyarginine, polyaspartates, polyornithine or saccharide derivatives thereof.

11. A liver retention clearing agent of Claim 10 wherein the liver retention component incorporates between about 3 and about 6 glutamic acid or lysine amino acids or galactosylated lysine amino acid derivatives.

12. A liver retention clearing agent of Claim 6 wherein the liver retention component incorporates at least three charged phosphonates of amino acids of natural (L) or unnatural (D) configuration.

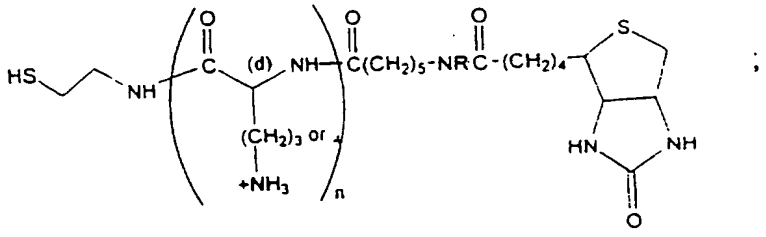
13. A liver retention clearing agent of Claim 12 wherein the liver retention component incorporates between about 3 and about 6 alpha phosphonomethyl amino acids of the formula:



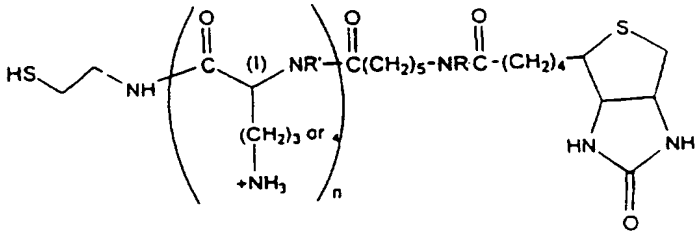
$$HOOC\text{-}CH(NH_2)CH_2\text{-}P(O)(OH)_2.$$

14. A liver retention clearing agent of Claim 6 wherein the liver retention component incorporates a neutral charge, hydrophilic disaccharide.

15. A liver retention clearing agent of Claim 1 wherein the liver retention component-binding component is one of the following:

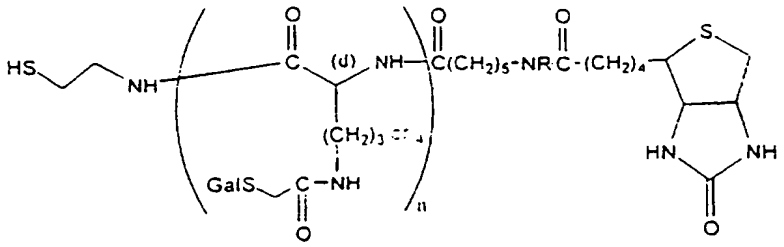


or

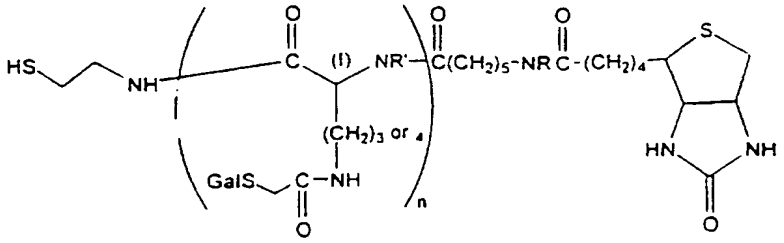


wherein n is about 3 to about 12; R and R' are lower alkyl groups from 1 to about 6 carbon atoms.

16. A liver retention clearing agent of Claim 1 wherein the liver retention component-binding component is one of the following:

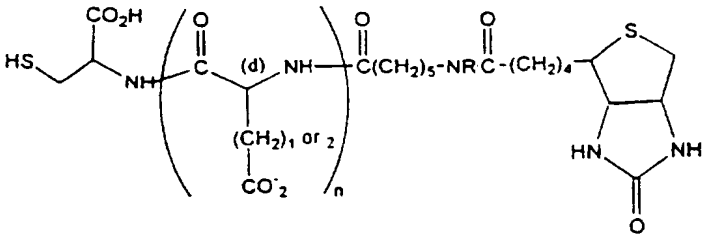


or

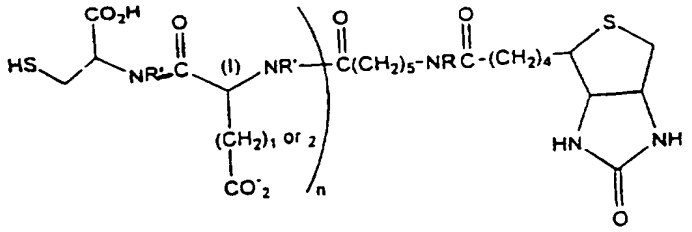


wherein n is about 3 to about 12; R and R' are lower alkyl groups from 1 to about 6 carbon atoms; and Gal is galactose.

17. A liver retention clearing agent of Claim 1 wherein the liver retention component-binding component is one of the following:



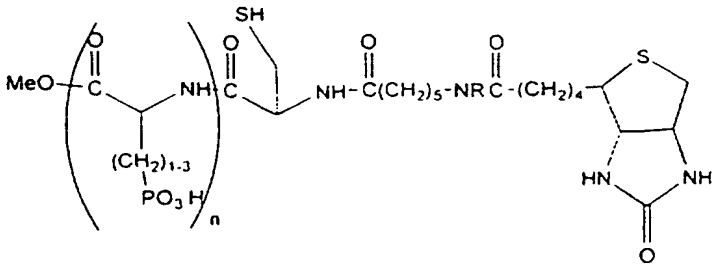
or

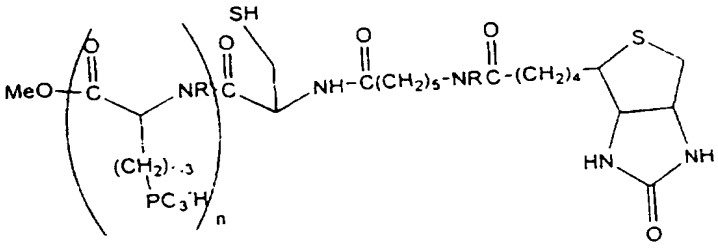


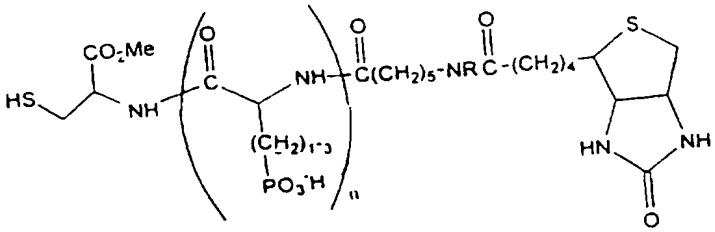
wherein n is about 3 to about 12; R and R' are lower alkyl groups from 1 to about 6

carbon atoms.

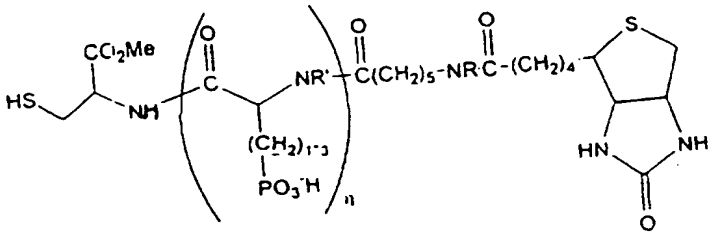
18. A liver retention clearing agent of Claim 1 wherein the liver retention component-binding component is one of the following:







or



wherein n is about 3 to about 12; R and R' are lower alkyl groups from 1 to about 6 carbon atoms.

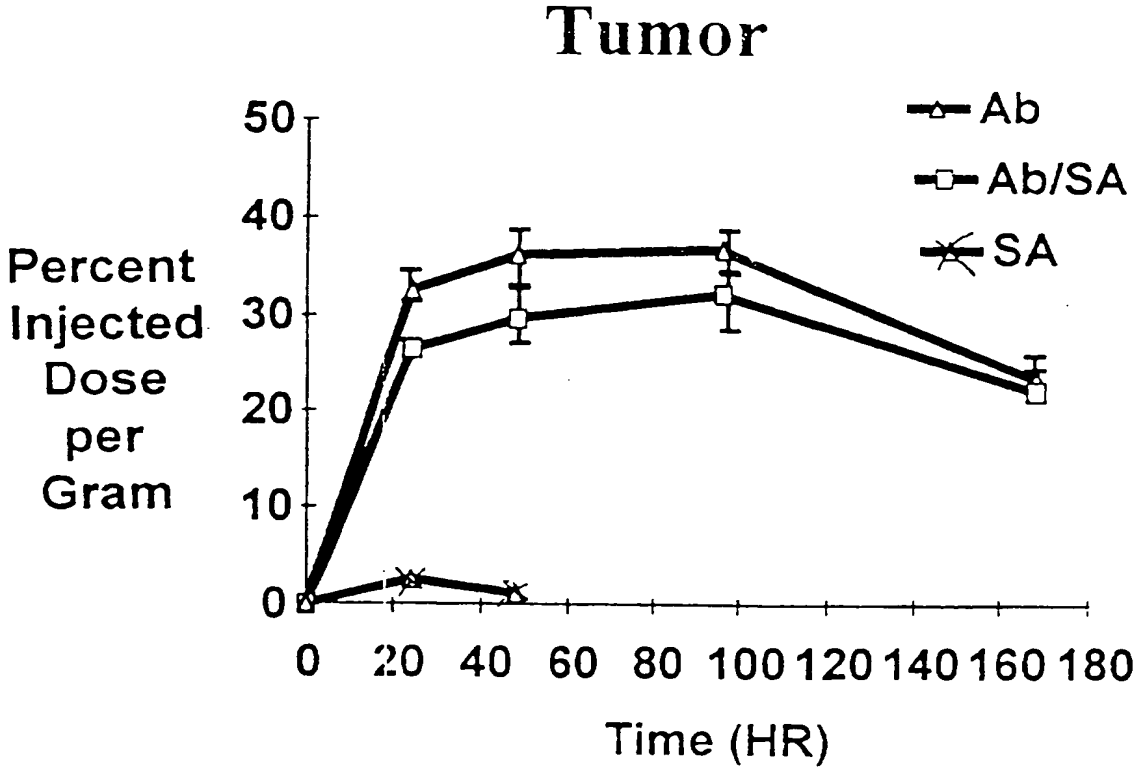


FIGURE 1

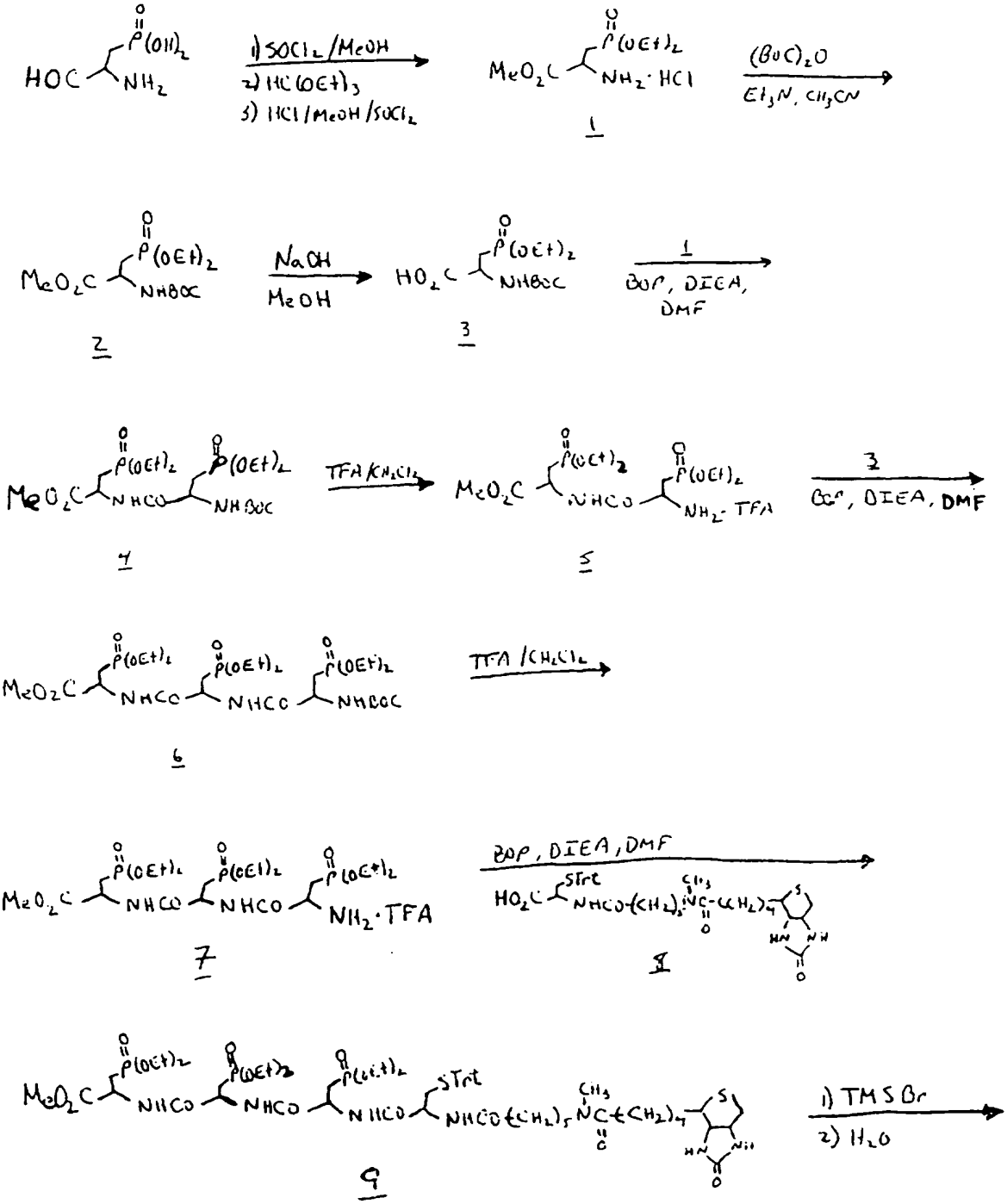
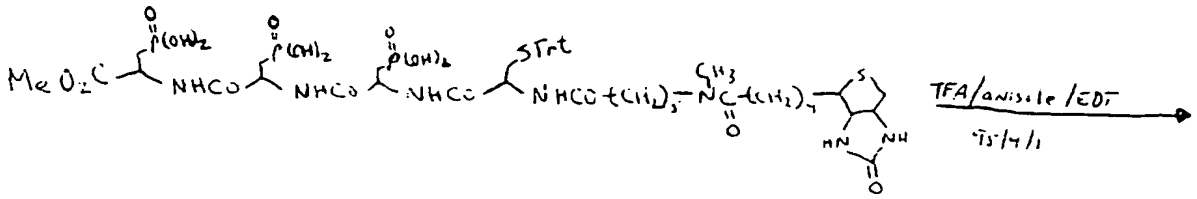

1) SOCl2/MeOH
2) HC(OEt)3
3) HCl/MeOH/SOCl2
(BOC)2O
Et3N, CH3CN
NaOH
MeOH
1
BOP, DIEA, DMF
TFA/CH2Cl2
TMSBr
H2O

FIGURE 2A



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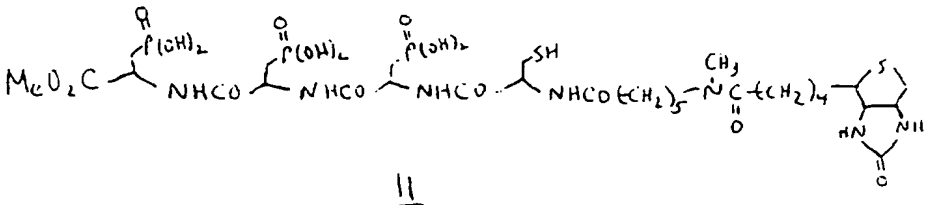

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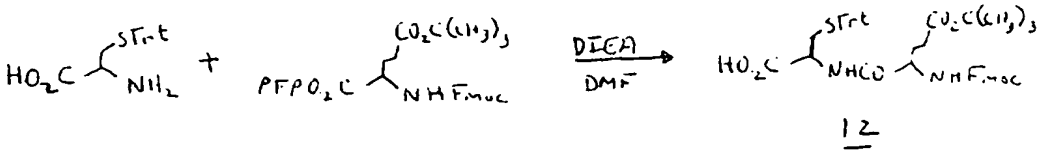
FIGURE 2B



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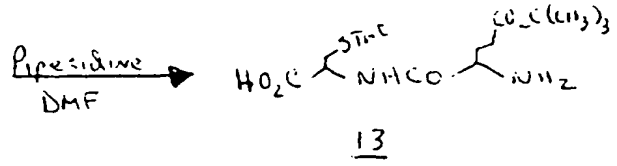

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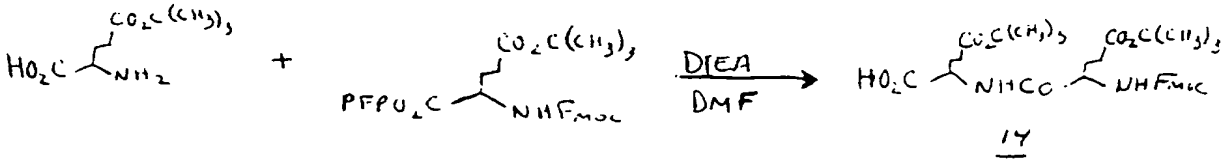

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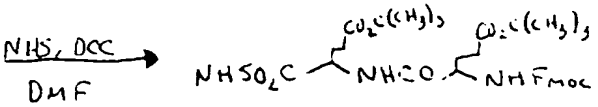

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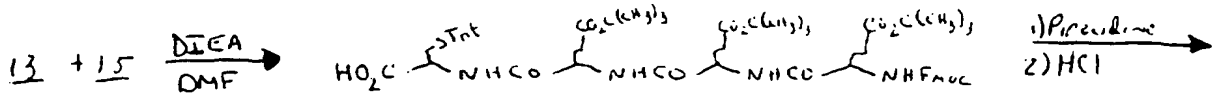

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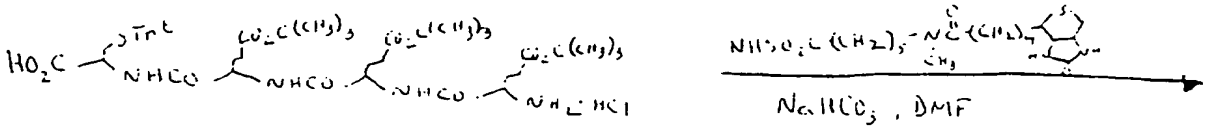
Figure 3A



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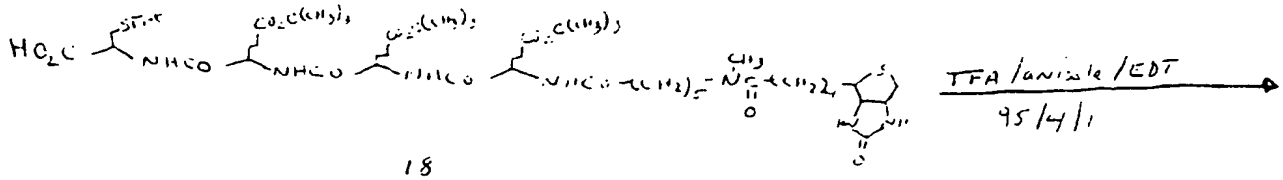
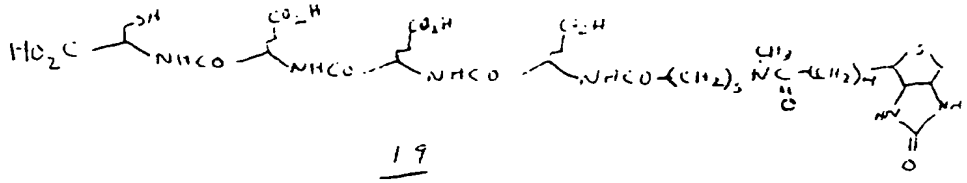


18



19

FIGURE 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09400

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 43/04; A61K 31/70
US CL :514/23; 536/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/23; 536/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dailog: USPATFULL, wpi, EMBase, Biosis. search terms: author and word (i.e. liver, hepativ, sugar, galactos?, sterochemistry, linker?, biotin, etc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MERWIN et al. Targeted Delivery of DNA Using YEE(GalNAcAH)$_3$, a Synthetic Glycopeptide Ligand for the Asialoglycoprotein Receptor. Bioconjugate Chem. 1994, Vol. 5, No. 6, pages 612-620, see entire document.	1-18
Y	BIESEEN et al. Synthesis of Cluster Galactosides with High Affinity for the Hepatic Asialoglycoprotein Receptor. J. Med. Chem. 1995, Vol. 38, No. 3, pages 1538-1546, see entire document.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 JULY 1997	14 AUG 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Heather Bakalyar, PhD Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09400

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VAN DER SLUIJS et al. Drug Targeting to the Liver with Lactosylated Albumins: Does the Glycoprotein Target the Drug or Is the Drug Targeting the Glycoprotein? Hepatology. 1986, Vol. 6, No. 4, pages 723-728, see entire document.	1-18

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